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Physiological Zoölogy

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FURTHER EXPERIMENTS ON THE ORIGIN OF THE ENTERIC NERVOUS SYSTEM IN THE CHICK[1]

(Twelve figures)

ERNEST VAN CAMPENHOUT
Department of Histology and Embryology, University of Montreal, Montreal, Canada

IN PREVIOUS papers (Van Campenhout, 1930*b* and 1930*c*, 1931), we have offered some evidence in favor of the thoracolumbar origin of the intestinal autonomic nervous system. This paper deals with the results of chorio-allantoic grafting experiments in which we have attempted to verify and to extend those conclusions and to correlate the embryological evidence with the physiological facts.

Chorio-allantoic grafting is a very fruitful method of studying the prospective potencies of embryonic regions or *anlagen*. Numerous papers by Willier (1924, 1930), by Willier and Rawles (1931), by Hoadley (1925), by Murray (1928), by Agassiz and Danchakoff (1922), and by a few other workers give clear evidence that a large number of embryonic areas undergo in these grafts a more or less normal development, frequently even a complete histological differentiation. Whenever an organ results from the interpenetration of two or more tissues arising either from *anlagen* originating far away or from chronologically distant sources, it will be easy to study the potencies of each tissue, isolated from the other normal constituents of the complex organ. Hence, in varying the ages of the donors

[1] The experimental work has been done in the department of anatomy of the Yale School of Medicine, New Haven, Connecticut.

and in taking well-located regions of the embryo, the origin and the rôle of these constituents in the formation of the adult organ can be determined.

The primary origin of the nervous ganglia in the walls of the digestive tract might be either one of the three following sources: the mesoblast, the neuroblasts migrating along the pneumogastric nerve, or those migrating along the sympathetic trunks. Little, if any, evidence speaks in favor of the mesodermic origin of the intestinal ganglia. Goormaghtigh (1924) and Tello (1925) were the most recent workers favoring this viewpoint, but in later papers they have modified their conclusions and have considered the ectodermic origin as the only possible one. In spite of the real weakness of the theory of the specificity of germ layers, and the few exceptions actually known to that theory, it should not be entirely discarded a priori. The other possible origins of nerve cells should be carefully looked for before admitting their differentiation *in situ*. Hasty conclusions, unwarranted by sufficient observations, seem to be responsible for the mesodermic theory (Camus, 1921; Dart and Shellshear, 1921). Whether the vagus nerve or the sympathetic nerve carries the neuroblasts which, by multiplication and extensive migration, are going to give rise to the nervous ganglia located in the walls of the digestive tract is more difficult to ascertain. The morphological and experimental observations we have presented in previous publications support strongly the orthosympathetic origin of the enteric nervous system, to the contrary of the classical literature (Kuntz, 1910, 1922; Kuntz and Batson, 1920; Müller and Ingvar, 1923; Abel, 1912; Uchida, 1927; and numerous other workers). It seemed, therefore, interesting to make further experiments in order to discriminate the vagal and the orthosympathetic contributions to the formation of these visceral ganglia.

Chorio-allantoic grafts will enable us to draw definite conclusions about the origin of the intramural nervous system of the digestive tract. Exact knowledge of the age of the donor will, of course, be necessary.

From the review of the embryological literature presented in a previous paper (Van Campenhout, 1930*a*) and from our morphological observations (Van Campenhout, 1931), we may safely con-

sider that at the end of the third day of incubation the pneumogastric nerves are very poorly developed. Their ganglionic crest is well formed; the nodosal ganglion is appearing; but there is not yet any trace of the visceral branch. This branch only appears around 86 hours of incubation; afterward it extends in the posterior mediastinum back of the esophagus and gives rise to a rich esophageal plexus and to the primary cardiac and pulmonary plexus. It extends as far as the esophageal end of the gastric dilatation around 96 hours and reaches the pylorus at the beginning of the sixth day (125 hours of incubation). The prevertebral sympathetic chains appear during the third day; and from the fourth day on, visceral branches extend ventrally and penetrate in the dorsal mesentery. The prevertebral abdominal plexus are formed at the end of the fifth day. Neuroblasts and nerve fibers appear for the first time in the walls of the stomach and the intestine around the 96th hour of development.

In taking these facts into consideration, it will be easy to take transverse sections of the trunk region in which the vagi could not possibly have extended. It is evident that the vagus elements have not reached any organ caudal to the heart up to 86 hours. In such grafts of transverse slices of embryo, the spinal cord and its orthosympathetic derivatives will be present. In another series we will take up the visceral mass, not including the central nervous cord, prior to or later than the beginning of the neuroblastic migration ventrally to the notochord.

This double series of experiments, carried with donors of various ages, will enable us to study the rôle played by the parasympathetic and by the orthosympathetic systems in the differentiation of the nervous plexus located in the walls of the gastrointestinal tract.

MATERIAL AND METHODS

The technique of chorio-allantoic grafting has been described by many authors; we have followed very exactly the indications of Willier, who had the kindness to demonstrate his technique to us. Fragments of embryo are grafted on the allantoic membrane of 9- or 10-day-old hosts, and very exactly on the bifurcation of a good-sized blood vessel. The host is placed back in the incubator, and its

development goes on in a more or less normal way. Six or 8 days later, prior to the involution of the allantois, the egg is opened and the graft is placed in fixing fluids.

The fixation always took place in Bouin's solution (picric acid, formalin, glacial acetic acid); this fixative is by far the best one for chick embryos, although Zenker's fluid with either formalin or acetic acid also gives good results. The trichromic stain of Masson (iron haematoxylin, ponceau, and anilin blue) and the straight iron haematoxylin, followed with eosin or erythrosin, were commonly used. A few grafts were preserved in ammoniated alcohol or in formalin, for silver impregnation, but this technique did not give any successful results.

In stating the age of the donor embryo, we always verified its normal development by noticing a few embryonic features such as the extension of the vascular area, the size and general form of the embryo, the stage of encephalic segmentation, and the appearance of the eyes, the heart, and the liver *anlagen;* this was to avoid mistakes in taking, as normal, an abnormally slowed-up or hastened embryo.

Forty-seven grafts were particularly concerned with this problem; thirty-three did develop successfully—about 70 per cent.

We will first describe a few typical examples of our series; a critical analysis of the results will be offered in a final chapter.

DESCRIPTION OF THE RESULTS

PART I. GRAFTS OF TRANSVERSE SECTIONS OF THE EMBRYO

Graft M 30

In a 50-hour embryo we isolated the lower thoracic region by two transverse sections, the cranial one being just caudal to the heart *anlagen*, the caudal one midway between the heart and the point of penetration of the umbilical vessels. It developed in the host for 7 full days, so that at the moment of fixation the graft was 218 hours old and measured about 12 mm. in diameter.

Figure 1 shows a low-power aspect of the graft, which is fortunately cut transversely to the craniocaudal axis of the donor embryo; and the various organs show up in a more or less normal relationship.

The spinal cord is well developed from the standpoint of both size and histological differentiation. The white and gray matters stand out clearly; the motor neurones are voluminous and multipolar; the ventral and dorsal roots are present; and the spinal ganglia appear normal. In most of the sections, more or less wide hemorrhagic areas are found, mainly near the extremities of the spinal-cord segment.

FIG. 1.—Embryo M 30. Microphotograph (by Dr. L. C. Simard). The nervous ganglia can be seen around the circular muscular layer of the intestine. ×20.

One common feature in practically all our grafts is the existence in the spinal cord of a variable number of tubular cavities which give more or less an aspect of syringomyelia. When these cavities exist in the gray substance, the adjacent cells form a palissadic limiting epithelium, similar to the lining epithelium of the ependymal canal. Some sections therefore appear as having many ependymal canals which do not communicate one with another and which are entirely closed.

The spinal cord is incompletely surrounded by a cartilaginous ring which is hypertrophied in some places and missing in others.

The digestive tract is represented by a well-differentiated stomach and some intestine. The stomach shows the differentiation of saccular glands; the circular muscle layer is thick, while the longitudinal one is poorly developed. Numerous nervous ganglia can be found between the two muscular layers. Owing to the poor differentiation of the longitudinal musculature, these ganglia are located just outside the circular layer. Besides the stomach, there is a short segment of intestine which also shows the normal development of the circular muscle layer, a poorly developed longitudinal layer, and the existence of numerous ganglia outside the circular layer. In Figure 1, in spite of the low magnification, these easily can be seen as dark spots forming a discontinuous band surrounding the gut. Study of the serial sections shows the connections between these visceral ganglia and the central nervous system. In following the spinal nerves, we can see the communicating rami going to more or less voluminous prevertebral ganglionic masses; from these ganglia cellulofibrillar branches extend to the intestinal ganglia.

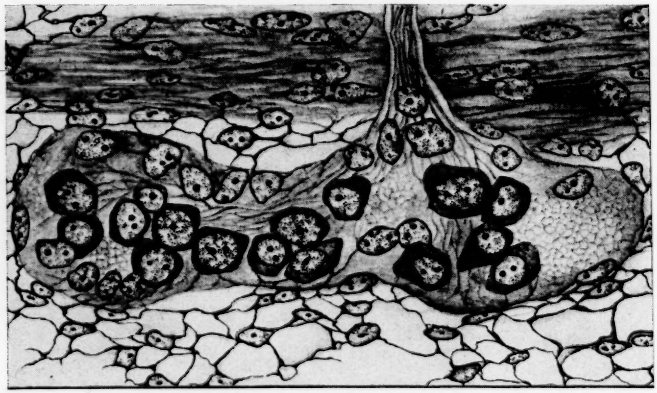

FIG. 2.—Embryo M 30. A myenteric ganglion sending a process in the circular muscular layer. × 585 (one-half the size of the original camera-lucida drawing).

The graft also shows a small amount of liver tissue, normally differentiated; intimately connected with it, there is a very small

cellular mass which looks like heart musculature. This heart rudiment is a compact organ, vascularized to be sure; but there is no cavity filled with blood. At the periphery of the graft, we find epithelial bands and vesicles, showing scores of mitoses and sometimes presenting the typical arrangement and structure of epithelial pearls.

Figure 2 shows a drawing of a typical myenteric ganglion, a branch of which penetrates deeply into the circular muscular layer; this branch seems to reach the submucosa. In Figure 3, we see ganglionic neuroblasts scattered in the musculature; these isolated nerve cells are found more or less deeply in the circular musculature, but we could not observe any such elements in the submucous layer.

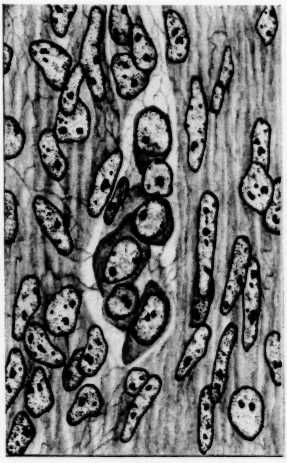

FIG. 3.—Embryo M 30. Neuroblasts in the circular muscular layer. × 585 (one-half the size of the original camera-lucida drawing).

Graft M 23

The thoracoabdomen of a 73-hour embryo was grafted on the allantoic membrane of a 9-day host. The graft was taken just above the umbilical pedicle; and its height, i.e., its craniocaudal length, did not measure more than 1 mm. Four and a half days later, it was killed, the graft being a total of 175 hours old.

Microscopic examination shows the graft cut longitudinally, nearly in a sagittal plane. The spinal cord therefore in some sections appears as a very long nervous band, from which the spinal roots originate. Hemorrhagic areas are less numerous and less extensive than in the preceding graft. The spinal ganglia exist in each segment and are voluminous.

The cartilaginous ring is complete, and centers of ossification can be observed. Ventrally to the vertebral column, the sympathetic chain can be seen, and the study of serial sections shows its continuity. Individual sections, however, seldom show it in more than two successive segments.

The digestive tract is represented by a sacculus and a long segment of intestine (Fig. 4). In the sacculus we find the differentiation of the characteristic glands, the two muscular layers, of which the longitudinal one is poorly developed, and the existence of numerous nervous ganglia and nerve trunks outside the thick circular layer.

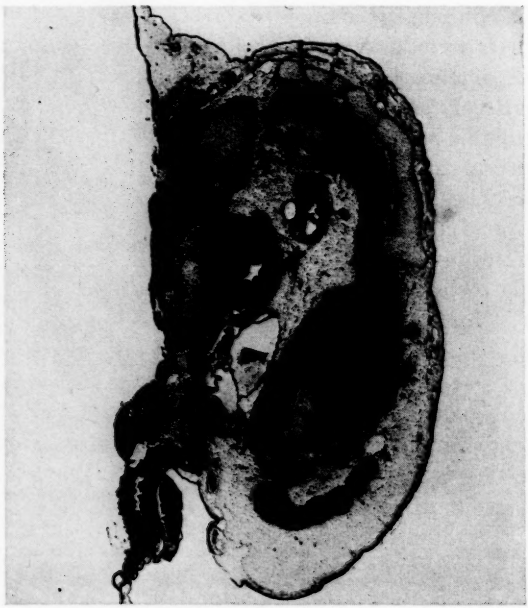

FIG. 4.—Embryo M 23. Microphotograph (by Dr. L. C. Simard). Two voluminous nervous ganglia outside the circular muscular layer of the intestine. × 20.

The intestine is well differentiated, but here also the longitudinal musculature is poorly developed. The nervous ganglionic masses are voluminous (Fig. 5); the neuroblasts are typically differentiated; but the amount of intercellular fibrillae is not as great as it was in the preceding embryo. The ganglia, in fact, seem to be somewhat younger than in M 30, and this is evidently supported by the differences in age between the two grafts. In following the series of sections, one can observe direct connections between the visceral gan-

glia and the prevertebral ganglia of the sympathetic chains. Numerous neuroblasts are still found along the connecting branches. Besides those myenteric ganglia, nervous cells are found deep in the intestinal circular musculature (Fig. 6). They are few in number, and they usually are grouped three or four elements together. Their appearance is quite characteristic and is extremely contrasting with the muscular or connective tissue elements. These interstitial cells

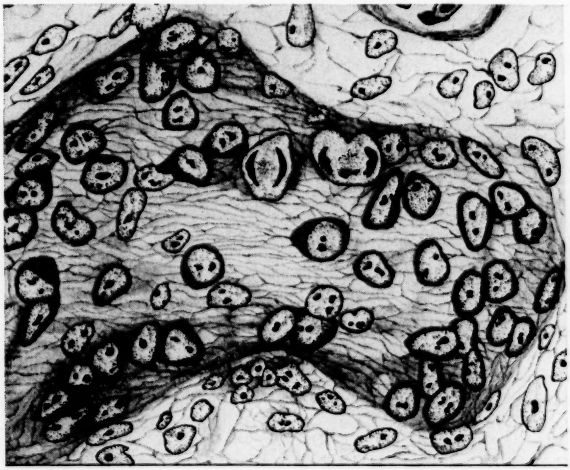

FIG. 5.—Embryo M 23. A myenteric ganglion. × 585 (one-half the size of the original camera-lucida drawing).

are found more or less near the submucosa, but we did not find any of them in this layer.

The liver is enormous and is connected with the intestine by means of a compact epithelial band, representing the pedicle which later on, by a hollowing process, is going to become the bileduct. Along this pedicle a few nervous ganglia are found; they are connected with the myenteric intestinal ganglia.

In the dense mesenchyme surrounding the intestine, there are a few epithelial lobules, which seem to represent a pancreatic *anlagen*. They are found just caudal to the liver pedicle. A few mesonephritic tubes exist more caudally and on both sides.

Graft M 26

In a 50-hour embryo, a graft was taken in the region of the umbilical pedicle, extending from 1 mm. cranial to 1 mm. caudal to it. It was kept on the allantoic membrane for 7 days, so that the total age of the graft was 218 hours.

The graft is cut transversely to the craniocaudal axis, and the topographic relationships of the various organs are about normal (Fig. 7). The spinal cord is well developed in size and in histological differentiation. The spinal ganglia are enormous; in the spinal canal they extend all along the lateral aspect of the cord; a ganglionic process passes between the successive cartilaginous rings, and a certain amount of ganglionic tissue is found outside the spinal cord. The prevertebral sympathetic chains are normally differentiated, and the solar plexus is represented by numerous nerve trunks and ganglionic masses. From these coeliac ganglia, numerous cellulofibrillar trunks extend toward the digestive tract and penetrate into the musculature. The digestive tract is represented by a long segment of intestine, which sends a ventrolateral diverticulum representing the umbilical pedicle. Along this diverticulum can be seen the umbilical artery and veins. The wall of the intestine shows numerous ganglionic masses. Some are found outside the musculature, just underneath the peritoneal serosa (Fig. 8). Many ganglia exist outside the circular muscle layer (Fig. 9). Numerous nerve cells are found between the layers of the circular musculature, and a few neuroblasts exist in the submucosa. In following the serial sections, one can observe the nerve trunks coming from the solar plexus and reaching those variously located ganglionic masses by means of more or less important fibrillar branches.

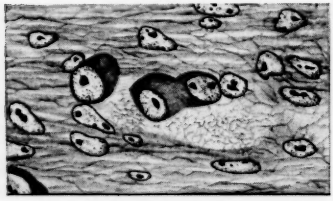

FIG. 6.—Embryo M 23. Neuroblasts in the circular muscular layer. X 585 (one-half the size of the original camera-lucida drawing).

The only other viscera represented in this graft are the mesonephric tubes, which are very numerous, particularly on one side. The Wolffian duct is present.

Graft M 21

The caudal extremity of a 72-hour-old embryo was grafted on the allantoic membrane of a 9-day-old embryo, and the fixation occurred when the total age of the graft was 199 hours.

The microscopic examination shows transverse sections of the

FIG. 7.—Embryo M 26. Microphotograph (by Dr. L. C. Simard). The intestine shows the origin of the umbilical pedicle. × 20.

graft (Fig. 10). The spinal cord is well differentiated and shows numerous hemorrhagic areas; dorsal root ganglia and spinal nerves are well developed. The vertebral cartilages are irregular in thickness and form more or less incomplete rings. Prevertebral ganglia are present, and from them arise a few branches extending toward the viscera. The digestive tract is represented by a segment of intestine which, from a histological standpoint, is less differentiated than the intestine in the preceding graft. The circular muscle layer is well developed, and outside this layer a few nervous ganglia can

be found; these ganglia are not as fully differentiated as those observed in M 26, but their nervous nature is quite certain (Fig. 11). They are directly connected with the prevertebral ganglia; in some sections the connecting branches form a plexus-like arrangement. The graft shows a large amount of mesonephric tissues.

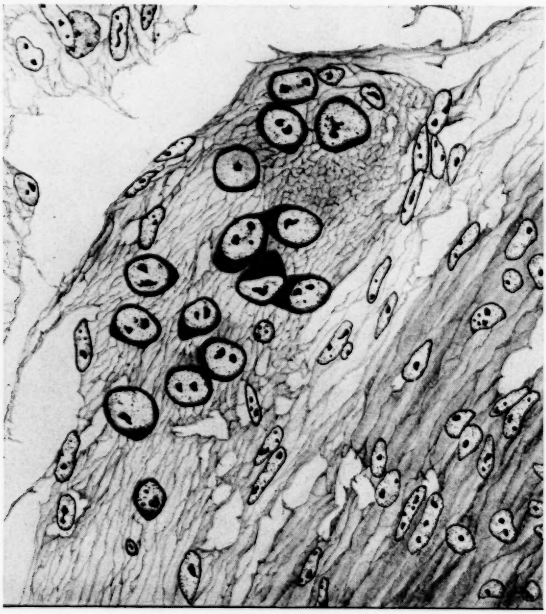

FIG. 8.—Embryo M 26. A subserous ganglion. × 585 (one-half the size of the original camera-lucida drawing).

PART II. GRAFT OF ISOLATED VISCERA

Graft G 3

In a 72-hour-old embryo, the intestinal loop has been dissected out and grafted on the allantoic membrane of an 8-day-old embryo. The graft was fixed at the total age of 189 hours; it was very small but well vascularized.

The microscopic examination shows the existence of an intestine (Fig. 12). The musculature is very poorly developed, there being

only traces of a circular muscle layer; the longitudinal layer is entirely missing. There are no traces of nervous elements either in the walls or in the surroundings of the digestive tract. The liver *anlagen* exists, and it shows a normal histological differentiation.

FIG. 9.—Embryo M 26. Microphotograph (by Dr. L. C. Simard). A myenteric ganglion. × 875.

There is no other organ present, excepting a small amount of cartilaginous tissue. Nervous cells or trunks could not be observed.

Graft G 9

The digestive tract was taken in a 192-hour-old embryo, from caudal to the heart to the umbilical pedicle. The graft was placed on the allantoic membrane for 6 days, thus reaching a total age of 336 hours.

The digestive tract is extraordinarily developed and shows a completely normal differentiation. It is represented by the sacculus, the

ventriculus, the small intestine, a voluminous liver, and a small pancreatic *anlagen*. Stomach and intestine show the existence of the two layers of musculature. Numerous nervous ganglia and nerve trunks exist between these two layers, forming a well-developed plexus of Auerbach. Many ganglia are found in the submucosa, close

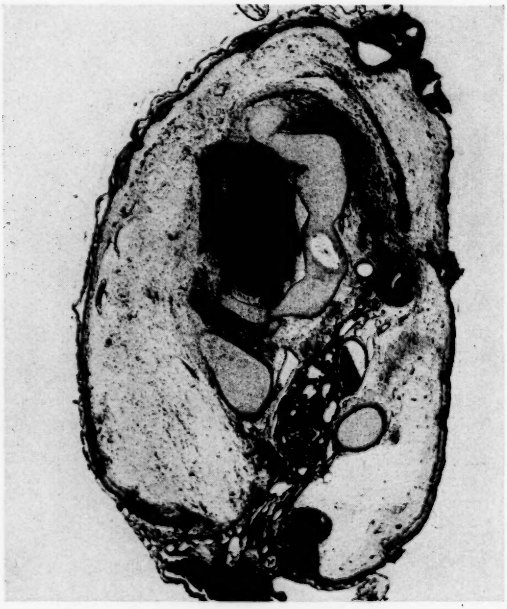

FIG. 10.—Embryo M 21. Microphotograph (by Dr. L. C. Simard). Very small intestine with incomplete circular muscular layer. × 20.

to the deep aspect of the circular muscular layer, representing a plexus of Meissner.

In the stomach, numerous nervous trunks and ganglia are found outside the longitudinal musculature. Small nervous ganglia exist around the pedicle of the liver and in the substance of the pancreas. A few small ganglionic masses are located in the surrounding tissue; fibrocellular trunks connect them with the visceral ganglia of the digestive tract. These trunks sometimes form a real plexus.

Further description of experimental cases would not offer any par-

ticular interest concerning the problem we are interested in. Such cases confirm the previously mentioned results, and we would have to repeat the same descriptions without any fundamental variations.

DISCUSSION

The conclusions of our experiments are easy to be drawn, and we will state them very briefly. The grafts M 21, M 23, M 26, and M 30

FIG. 11.—Embryo M 21. Microphotograph (by Dr. L. C. Simard). A myenteric ganglion. × 875.

have been taken from embryos in which the vagus nerves could not possibly have reached the level of the transverse section used as graft. Therefore the intravisceral nervous ganglia and trunks found in the walls of the stomach and the intestine cannot be depending upon a migration of neuroblasts along the pneumogastric nerves. The grafts show the existence of subserous ganglia, of myenteric ganglia forming a well-developed plexus of Auerbach, and of neuro-

blasts migrating more or less deeply into the circular muscular layer. A few of these isolated ganglion cells could be found in the submucosa, so that the neurons of the Meissner plexus evidently result from a migration of neuroblasts through the circular musculature. This migration seems to start around the 218th hour of development; younger grafts do not show it. Whether all the neuroblasts found in the circular musculature reach the submucosa, or whether a few remain as interstitial cells in this musculature, will have to be studied. From the existence of these various sets of ganglionic cells in the walls of the digestive tract, we may safely conclude that the vagus nerves and the neuroblasts migrating along them are not necessary to the formation of a normal visceral autonomic nervous system in the gastric and intestinal segments of the digestive tract in chick.

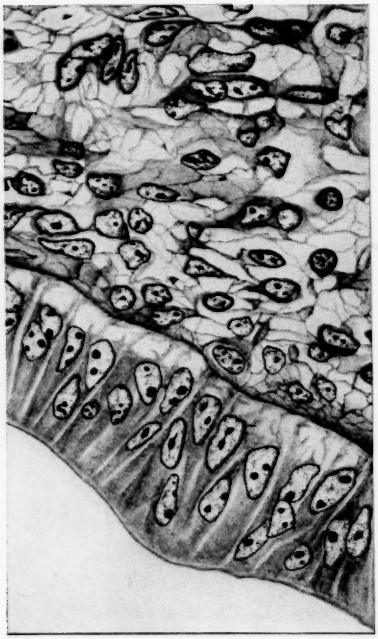

FIG. 12.—Embryo G 3. Intestine without intravisceral nervous ganglia. × 585 (one-half the size of the original camera-lucida drawing).

Grafts of intestine isolated from any central nervous system connection do not show the differentiation of visceral ganglia prior to the migration of neuroblasts into the viscera or their mesentery. The origin of visceral ganglia from mesoblast is therefore highly improbable. It is difficult to eliminate entirely the possibility of a differentiation of neuroblasts from mesodermic cells as a result of a special stimulation by connection or contact with sympathetic cells which have migrated into that mesoblast. However, all the evidence we have speaks strongly against such a far-fetched hypothesis.

The orthosympathetic origin and nature of the visceral autonomic system of the stomach and intestine cannot be denied.

In a previous paper (Van Campenhout, 1931), we have emphasized the fact that the pneumogastric nerves played the main part in the primary constitution of the autonomic nervous system of the heart, the lungs, and the esophagus; the descriptions of Kuntz were fully confirmed concerning these points. Our results, however, disagree with the conclusions of Kuntz and of most other workers concerning the innervation of the stomach and of the intestine. According to them, the cellular elements of the plexus in the walls of the entire digestive tract down to the end of the small intestine represent neuroblasts which have migrated along the branches of the vagi. The experiments described above do eliminate the possibility of such a vagal origin. The rôle played by the vagi in the enteric innervation is not of primary embryogenetic importance, and it only becomes evident in the late stages of histogenesis. The vagus nerves supply a great number of fibers to the walls of the stomach; these gastric fibers reach the pyloric region, but they do not seem to cross the pylorus. In old embryos, later than 8 days, the vagi send a few branches to the solar plexus. Whether these fibers pass through the plexus and by that route enter the mesenteric nerves, or whether they connect synaptically with the orthosympathetic neuroblasts of the coeliac ganglia, is unknown. The mesenteric nerves therefore can be considered either as containing both vagal and sympathetic fibers or as containing fibers from sympathetic neuroblasts which in the coeliac plexus are connected exclusively or mainly with vagal or sympathetic branches. The vagal or the sympathetic connection in the coeliac plexus will necessarily provoke differences in functional activities, although there is no actual difference in the nature or the origin of the nerve cells themselves. It is improbable that the vagus nerves would pass through the coeliac plexus without presenting a few connections with the sympathetic neuroblasts of the prevertebral ganglia. Therefore, the mesenteric nerves might be considered as a complex of four sets of fibers coming respectively from the vagus nerves, from the splanchnic nerves, from neuroblasts connected with preganglionic vagus fibers, and from neuroblasts connected with the sympathetic preganglionic fibers.

From a physiological standpoint, the recent investigations of Nolf (1928) represent the most careful analysis of the intestinal nervous system in the chick; we will summarize briefly a few outstanding results. Electric stimulation of the mesenteric nerves of an isolated segment of intestine has, at the same time, motor and inhibitory effects. Stimulation of the peripheral stump of the previously cut pneumogastric nerves gives rise to rhythmic contractions in various segments of the gut. Stimulation of the splanchnic nerves (primary coeliac nerves) causes motor and inhibitory phenomena. In using the nicotin test, Nolf shows that the inhibitory fibers of the mesenteric nerves are all postganglionic; that their stimulation is just as effective after section and degeneration of the vagi; and that these postganglionic inhibitory fibers therefore belong to the orthosympathetic, and their cell bodies lie in the prevertebral, ganglia. The mesenteric nerves also contain numerous preganglionic fibers, both vagal and sympathetic; their cell bodies are in the spinal cord. All these fibers synapse with the connecting neurons, which themselves send fibers to motor or inhibitory ganglionic cells in the walls of the intestine. The reaction, motor or inhibitory, will depend upon the state of the terminal nervous endings, upon the arterial blood pressure, and upon the intensity of stimulation.

Two facts are of particular interest in these physiological results: Nolf admits that there are sympathetic neuroblasts in the enteric nervous system and that the inhibitory sympathetic fibers are postganglionic. The enteric nervous system cannot be considered any more as a pure parasympathetic outflow, even from a physiological standpoint.

A complete morphophysiological investigation of the coeliac plexus is urgently needed for the understanding of the experiments dealing with the abdominal viscera. It is not because stimulation of the mesenteric nerves sometimes has inhibitory effects, while other times it stimulates or accelerates reactions, that one is allowed to conclude anything concerning the nature of the fibers in those mesenteric nerves. Similarity or differences in responses do not necessarily imply identity of, or differences between, fibers; differences in terminal connections are just as well responsible for opposite or different effects. In other words, anatomical classification and conclusions

based on physiological experiments would carry a much greater weight with some morphological support; this support is actually entirely missing.

The pure vagus nerve for a physiologist is an entirely different thing from the vagus nerve of an anatomist. The name of "tenth cranial nerve" can only be given to the intracranial part of the pneumogastric nerve. As soon as it reaches the ganglion nodosum, it receives and gives many connecting branches and perhaps cells from or to the nearly adjacent superior cervical sympathetic ganglion. This fact has been shown by numerous authors, and recently by Terni (1923, 1925), Uchida (1927), and this author (1931). The anastomoses show up very clearly in a gross dissection.

From there on, the vagus nerve is a vagosympathetic trunk, and we do not know whether the proportion of vagal and of sympathetic components remains the same throughout the entire length of this trunk or whether the trunk becomes more vagal or more sympathetic as it extends caudalward. Vagal effects should mainly be sought for in working on the supranodosal segment of the tenth cranial nerve.

Among many instances this illustrates the fact that a direct collaboration between morphologists and physiologists is the only way in which further successful investigation of the autonomic nervous system is to be carried out. As long as we consider separately the physiological reactions and the anatomical facts, we will not understand the histophysiology or the functional anatomy of the enteric nervous system.

CONCLUSIONS

1. The origin and the complete differentiation of the intravisceral gastric and intestinal nervous plexus are independent of the pneumogastric nerves.

2. The ganglionic cells of the enteric nervous system represent orthosympathetic elements with which the vagus nerves get in direct or indirect connection during late stages of development.

3. In the stomach, the pneumogastric fibers extend outside the longitudinal musculature, and they probably connect directly with the orthosympathetic neurones of the Auerbach or Meissner plexus.

4. Whether the vagus fibers connect synaptically in the coeliac ganglia with orthosympathetic ganglion cells or whether they simply

pass through the solar plexus toward the mesenteric nerves is an open question, the solution of which is necessary for the understanding of the mechanism through which the pneumogastric nerves influence the intestine.

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THE GROWTH AND DIFFERENTIATION OF DUCK KIDNEY TISSUE ON THE CHORIO-ALLANTOIC MEMBRANE OF THE CHICK AND DUCK

(Two plates)

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THERE is considerable evidence that transplantations made between individuals of different species are often successful if done during the embryonic stages. This has been demonstrated for the early stages of amphibian development through the investigations of Spemann and Mangold (1924), Geinitz (1925), Mangold and Seidel (1927), Bytinsky-Salz (1929), and other workers of the Spemann school. Harrison (1898) and Morgan (1899) succeeded in grafting two species of *Rana* during the later embryonic stages. Among the higher vertebrates, Murphy (1913, 1914*a*, 1914*b*, 1916, 1926) was able to grow hashed embryonic and sarcomatous tissues of the rat on the chorio-allantoic membrane of the developing chick embryo. Using the same method of chorio-allantoic grafting, Hiraiwa (1927) demonstrated that certain rat tissues are capable of growth and differentiation when sections of 11-day-old embryos are transplanted.

On the other hand, transplants between adults of different species are usually unsuccessful. Loeb (1920, 1921), Loeb and Harter (1926), Fleisher (1920, 1921), and a host of others have made extensive studies of transplants involving adults of variously related animals, especially mammals. In general, they demonstrated a definite type of cellular reaction consisting of an invasion of lymphocytes and granulocytes, and the growth of connective tissue in and around the grafts of foreign tissue. According to Loeb and Wright (1927), the intensity of this reaction seems to be correlated with the degree of relationship between the animals involved.

From the foregoing evidence it is quite obvious that during the

development of vertebrates a condition arises which makes it impossible to graft tissues from one species to another; i.e., a species specificity develops. With the exception of Murphy and Loeb, few workers have been concerned with the initial occurrence of this specificity. Murphy (*loc. cit.*) found that hashed embryonic and sarcomatous tissue of the rat grew well on the chorio-allantoic membrane of the chick until the host had been incubated for 18 days, at which time a specificity developed that was expressed by a lymphocytic reaction against the foreign tissue. Loeb (1925), without referring to any specific experimental evidence, explains the incomplete reaction in embryonic growth, as compared to that in the adult, by "primary or secondary deficiency in genes, simplification in structure of the individuality differential, diminished secretion of the specific substances on the part of the transplants, and increasing resistance on the part of the host."

Because very little study has been attempted on the development of species specificity in the developing embryo, a more complete analysis was started. The experiments were originally planned to determine when in the process of differentiation the duck kidney tissue became species specific. In a preliminary report (Sandstrom, 1929) it was concluded from a series of transplants of variously aged duck kidney tissue made to the chorio-allantoic membrane of the chick that a species specificity developed in the donor tissue between the twenty-fourth and twenty-seventh days of incubation. Subsequent experiments, however, have cast doubt on this conclusion. The present paper, an outgrowth from the original series of experiments, is concerned with the capacity of metanephric tissue of the duck to grow on the chorio-allantoic membrane of the duck as well as the chick. However, additional experiments are in progress; and others are planned, involving different tissues of the duck and also various tissues of other classes of vertebrates, with the hopes of obtaining more conclusive data on the original problem, namely, the relation of the time of the appearance of species specificity to differentiation.

This investigation was originally suggested to me by Dr. B. H. Willier, of the University of Chicago; and the experiments included in this paper were carried out under his direction at the Hull Zoölogical Laboratory. To him I owe my sincerest appreciation for sug-

gestions and criticisms offered throughout the course of the work and preparation of the manuscript.

MATERIAL AND METHODS

The method of chorio-allantoic grafting used here was essentially the same as that described by Willier (1924). Briefly stated, it consisted in removing the tissue from the donor and placing it in normal salt solution, kept at a temperature of approximately 38° C., where it was cut up into pieces ranging from 1 to 4 cubic millimeters. The chorio-allantoic membrane of the host embryo was then exposed by sawing a rectangular window in the shell at a location above the junction of two of the larger blood vessels, a position previously determined by candling. A bit of the tissue was then placed on the exposed chorio-allantoic membrane by means of a needle, and the egg again sealed by replacing the piece of shell and painting the cut edges with melted paraffin. All this was done under fairly aseptic conditions. In all instances the duck tissue was implanted on the chick host embryo during the ninth day of incubation, and on the duck host embryo during the fourteenth day of incubation, the ages found most suitable for the reception of the implant; and after another period of incubation lasting eight days, the grafts were removed. Histological study then followed. Either Bouin's or Zenker's fixatives were used. The tissue was imbedded in paraffin, sectioned serially at 8 μ, and stained either in iron-haematoxylin or eosin-methylene blue.

The duck was selected as a source of foreign tissue because it can be incubated under conditions identical with those required by the chick. As a check, successful hatchings of ducklings were obtained from eggs incubated in the same incubators with the host embryos. Thus the temperature factor which seems to have played a part in the experiments of Hiraiwa (1927) when he transplanted rat tissue to the chick has been avoided. The duck was selected also because it was believed to be sufficiently distantly related to the chick to insure a decided cellular reaction. Throughout the experiments, the embryos of white Leghorn chickens and white Pekin ducks were used as hosts and white Pekin ducks as donors.

Of all the tissue available in the donor, the kidney was selected for

the preliminary experiments because Atterbury (1923) has shown that, in the chick, it is capable of complete independent embryonic differentiation. She isolated the primordium at a very early stage and grafted it to the chorio-allantoic membrane of another embryo. It developed to an apparently functional stage which appeared histologically normal. That a similar condition existed for the duck was assumed, and the results of the experiments reported in this paper indicate the validity of the assumption. Furthermore, the metanephros is simple in its embryonic formation being composed, with the exception of innervation, of tissues derived wholly from the mesoderm. This eliminates the possibility of any complications which might be expected from structures composed of derivatives of more than one germ layer. The metanephric tissue was obtained from duck embryos ranging in age from 13 to 28 days; from ducklings, from 29 to 38 days; and from adult ducks.

The controls for these experiments were of two types: First, the normally developed metanephroi from duck embryos whose ages corresponded to those of the grafted tissue at the time of implantation and removal were used as controls for the transplants of duck kidney tissue made to the duck and the chick. Second, the transplants of duck kidney tissue made to the duck were used as controls for the transplants made to the chick.

RESULTS

In general, the results of the transplantations of metanephric tissue from the variously aged duck donors made to the chorio-allantoic membrane of the chick are indicated in Table I. In this table are summarized all the data capable of tabulation,[1] the remaining data being included in the descriptions of the histological structure of the grafts.

Referring to the table, it will be noted that the amount of apparently normally differentiated tissue in the grafts after growing on the chick membrane for 8 days is at a maximum in those cases

[1] In order to tabulate conditions of grafts for superficial comparison, positive (+) and negative (−) signs in various combinations have been used. They are based on estimates made after carefully studying the preparations, but are not intended to record information based on actual measurements.

TABLE I

GRAFTS OF DUCK METANEPHRIC TISSUE ON CHORIO-ALLANTOIC MEMBRANE OF CHICK

SERIES	CASE NO.	AGE OF HOST (DAYS)	AGE OF DONOR* (DAYS)	TIME ON HOST (DAYS)	TOTAL AGE OF GRAFTED TISSUE (DAYS)	SIZE OF GRAFT AT THE TIME OF REMOVAL (MILLIMETERS)	NORMAL TISSUE	NECROTIC TISSUE	CELLULAR REACTIONS			REMARKS
									Connective Tissue	Lymphocytes	Granulocytes	
I....	61-28	9	13	8	21	5.32×1.90×3.90	++++	−		−	+++	
	61-36	9	13	8	21	6.10×2.30×5.28	++++	−		−	+++	
	61-37	9	13	8	21	3.00×1.60×3.36	++++	−		−	+++	
	62-15	9	16	8	24	3.03×3.00×1.40	++++	−		−	+++	
	62-16	9	16	8	24	0.50×1.80×1.20	++++	−		−	+++	
	62-20	9	16	8	24	3.75×2.90×1.30	++++	−		−	+++	
II....	63-29	9	19	8	27	2.36×1.90×1.71	+++	+	+	−	+++	
	63-31	9	19	8	27	1.56×1.10×1.70	+++	+	++	−	++	
	82-1	9	19	8	27	1.40×1.00×1.26	+++	+	++	−	++	
	82-3	9	19	8	27	1.03×1.20×0.60	+	++	+	+	++	
	82-5	9	19	8	27	0.90×1.20×0.60	+++	+	−	−	++	
	82-11	9	19	8	27	0.60×0.40×0.96	−	++++	++	−	−	
	82-16	9	19	8	27	1.41×1.30×1.00	+++	+	−	−	+	
III...	64-32	9	21	8	29	.....	−	++++	−	−	++	†
	64-37	9	21	8	29	.....	−	++++	++	−	++	†
	65-4	9	24	8	32	No tissue present	.....	.....	+++++	+	++	
	65-5	9	24	8	32	No tissue present	.....	.....	+++++	−	++	
	65-7	9	24	8	32	No tissue present	.....	.....	+++++	+	+	
	65-8	9	24	8	32	No tissue present	.....	.....	++++	−	++	
	65-15	9	24	8	32	0.66×0.70×1.10	−	++++	++	−	++	
	65-19	9	24	8	32	No tissue present	.....	.....	++++	−	+	
	65-21	9	24	8	32	.....	−	++++	+	+	−	†
	65-24	9	24	8	32	.....	−	++++	+	+	+	†
	66-32	9	26	8	34	2.70×1.40×1.90	+	+++	+++	+	++	
	66-37	9	26	8	34	1.70×1.00×1.78	++	++	+++	−	++	†
	66-39	9	26	8	34	.....	−	+++	−	−	−	†
	66-42	9	26	8	34	1.60×0.70×1.39	+	+++	+++	−	++	†
	66-43	9	26	8	34	.....	−	+++	++	+	++	†
	55-1	9	27	8	35	1.00×1.30×1.90	+	+++	+++	−	+	
	55-27	8	27	8	35	.....	−	+++	+++	+	++	†
	55-30	9	27	8	35	.....	−	++++	+	−	+	†
	67-7	9	28	8	36	0.80×0.90×1.74	−	++++	++++	−	+	
	67-8	9	28	8	36	2.20×1.20×0.50	+	+++	+++	−	+	
	67-32	9	28	8	36	0.40×0.50×2.21	+	+++	+++	−	++	
	67-34	9	28	8	36	0.40×0.50×0.48	−	++++	+++	−	+	
	67-39	9	28	8	36	0.40×1.10×1.28	−	++++	++	−	+	§
	67-40	9	28	8	36	0.80×1.10×1.21	+	+++	+++	+	+	
IV...	50-15	9	35	8	43	0.94×0.60×0.80	++	++	+++	−	++	
	50-16	9	35	8	43	0.90×1.00×1.19	+	+++	+++	−	+	
	50-17	9	35	8	43	1.00×0.80×1.20	+	+++	+++	−	+	
	50-19	9	35	8	43	0.10×0.70×1.32	−	++++	+++	−	+	
	50-22	9	35	8	43	1.43×0.80×0.50	−	++++	+++	−	+	
	50-23	9	35	8	43	0.07×0.40×0.70	−	++++	+++	−	−	§
	50-24	9	35	8	43	1.00×1.10×0.80	+	+++	+++	−	−	
	53-29	9	35	8	43	1.38×1.40×0.80	−	++++	+++	+	−	
	53-33	9	35	8	43	0.98×1.10×0.70	+++	+	+	−	+	
	51-6	9	38	8	46	1.00×0.30×0.06	+	+++	+	−	+	
	51-28	9	38	8	46	0.60×0.30×0.28	−	++++	++	−	+	
V....	45-5	9	Adult	8	Adult	0.73×0.60×0.50	−	++++	++	−	−	
	45-30	9	Adult	8	Adult	1.49×1.00×0.60	−	++++	+++	−	−	
	46-2	9	Adult	8	Adult	0.54×0.60×0.30	−	++++	++	−	+	
	46-3	9	Adult	8	Adult	1.00×1.10×0.50	−	++++	+++	−	−	
	46-8	9	Adult	8	Adult	1.23×1.20×0.90	−	++++	+++	−	+	
	46-11	9	Adult	8	Adult	0.70×0.60×0.88	−	++++	+++	−	−	

* The age of the donor is given as the number of days since the initiation of incubation. The period of incubation for the duck is 28 days; hence, in Series III and IV the age indicates the period of incubation plus a certain number of days of extra-shell life, e.g., 35 days = 28 days of incubation + 7 days of extra-shell life.

† Grafts so small or scattered that they could not be measured, and the size is merely estimated to be smaller than 1 cubic millimeter.

§ Sloughing.

grouped in Series I. In this series are included all the transplants, the donors of which were at 13 and 16 days of incubation. The sizes of the grafts show a large increase over the sizes of the pieces originally transplanted, indicating an active growth. All (++++) of the tissue in the grafts is normal, and there is a complete absence (−) of necrosis. Infiltrations of connective tissue and lymphocytes are absent (−), but granulocytes are abundant (+++).

Series II includes all of the transplants made from metanephric tissue of duck embryos at 19 days of incubation. Here the increase in size of the graft over the original piece transplanted is not so evident, but some growth has taken place. Necrosis, however, is present, ranging from small (+) areas to almost complete (+++) necrosis; and there is a corresponding decrease in the amount of normal tissue. Accompanying the necrotic condition of the transplanted tissue, there is usually some evidence of an infiltration of connective tissue, the intensity and quantity of which varies (+ to ++) in direct proportion to the amount of necrosis. Lymphocytes are never very abundant, although in some cases they appear to be slightly increased over the number normally present in the host (+). Granulocytes are less common that in the preceding group.

Series III includes all of the transplants made from duck donors ranging in age from 21 to 28 days of incubation. The sizes of the grafts indicate clearly that there has been no growth of the tissue while on the chick membrane; in many cases a decided decrease in size is evident. Usually very little (+ to ++) normal tissue appears in the grafts, while necrotic tissue is quite extensive, ranging from partial (++) to complete (++++) necrosis. In some cases the grafted tissue has been absorbed, and connective tissue has completely (++++) replaced it. In all other cases the connective tissue varies from a slight (+) increase to an extensive invasion (+++). Lymphocytes are never very abundant, and granulocytes are less evident.

All transplants made from duck donors varying in age from 35 to 38 days (7–10 days extra-shell life) are included in Series IV. The sizes of the grafts show, without exception, a reduction in size, indicating that an absorption of the transplanted tissue has taken place. More of the tissue is necrotic than in the previous group, al-

though occasionally some normal tissue is found in some of the grafts. The amount of connective tissue, as a rule, increases with the increased necrotic tissue; but both lymphocytes and granulocytes are noticeably absent.

Series V includes all grafts made from kidney tissue of adult ducks. The sizes of the grafts indicate absorption. The grafts are completely necrotic, and connective tissue is abundant. Lymphocytes and granulocytes, as in Series IV, are no more abundant than normally present in the host.

A. STRUCTURE OF THE DUCK KIDNEY AT THE TIME OF TRANSPLANTATION

The normal metanephros of the duck on the thirteenth day of incubation, the earliest stage transplanted, is approximately 4 millimeters long. Histological preparations reveal both collecting and secreting tubules formed in the central part of the organ. At the periphery, closely massed about the ends of the collecting tubules which are derived as branches of the original metanephric diverticulum, nephrogenous tissue is being transformed into secreting tubules. The collecting tubules are readily recognized by their cuboidal cells. The cytoplasm is clear and distinctly basophilic, coloring a pale blue when stained with eosin-methylene blue. The nuclei are centrally situated in the cells. The secreting tubules are easily distinguished from the collecting tubules by their larger and slightly pyramidal cells and very granular cytoplasm, which stains a faint pink in eosin-methylene blue preparations. The nuclei are slightly displaced toward the proximal ends of the cells. Mitotic figures are abundant in the cells of both kinds of tubules. At this stage glomeruli have formed in only the more differentiated central regions of the metanephros and are not common. The spaces between the metanephric structures are filled with a loose mesenchyme in which there are located groups of granulocytes.

At the twenty-first day of incubation the number of tubules of the normal kidney is greatly increased. The peripheral undifferentiated nephrogenous tissue is converted almost entirely into secreting tubules. Mitotic figures are still very numerous in the cells. The glomeruli are many. The interstitial connective tissue is greatly decreased, and granulocytes are not as common. At the time of hatch-

ing, namely, the twenty-eighth day, the kidney is practically differentiated except at the periphery, where isolated masses of nephrogenous tissue still persist. It is found in lesser quantities, the older the structure, and is probably entirely differentiated into functioning structures in the adult.

B. GRAFTS OF DUCK KIDNEY TISSUE MADE ON THE CHORIO-ALLANTOIC MEMBRANE OF THE CHICK

Series I. Donors at 13 and 16 days of incubation.—The histological structure of the transplanted duck metanephric tissue, after growing on the chorio-allantoic membrane of the chick host, is essentially the the same for all of the eight grafts in the series, and differs in no respect from the normal kidney of comparable age (Plate I, Fig. 1). It is possible that there may be a slight inhibition in the development due to insufficient vascularization in the early stages of incorporation into the host membrane, but this is so slight as to be scarcely recognizable. There is no evidence of necrosis of the transplanted tissue. The secreting and collecting tubules are normal. Their cellular structure, when compared with that of the tubules of the normally developed organ, shows no effect from the heteroplastic grafting; and the mitotic figures are just as numerous. Glomeruli are equally as abundant and as well formed.

There is no evidence of a cellular reaction by the host against the foreign tissue. The grafts, as a rule, are well incorporated into the chorio-allantoic membrane. The connective tissue immediately surrounding the duck kidney tissue is normal, and there are no signs of either a proliferation or an infiltration into the grafted tissue. Lymphocytes are no more abundant than would be expected in any other part of the chorio-allantoic membrane of a 17-day-old chick embryo. Granulocytes are abundant in the interstitial tissue, but they also occur in the normally developed organ. They seem to be somewhat more abundant in the graft, however, than in the normal structure. All of the grafts are well vascularized by the large blood vessels that are common to the chorio-allantoic membrane.

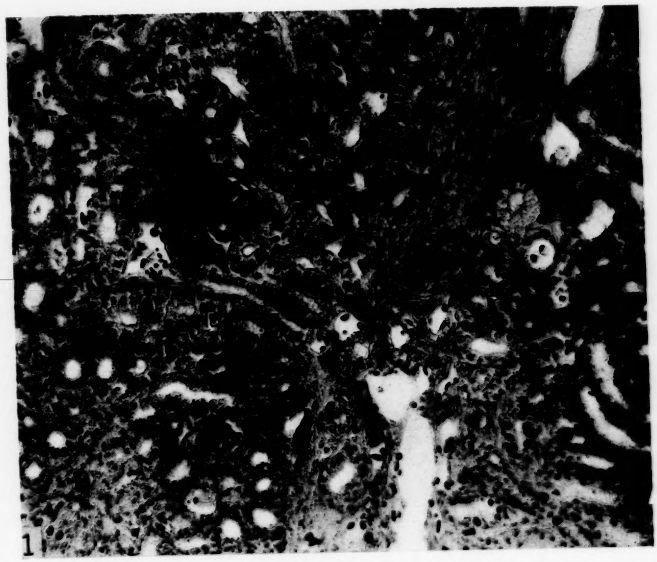
Series II. Donors at 19 days of incubation.—Histological preparations of these grafts show that in all cases, except Case No. 82-11, there are indications of necrosis and an accompanying cellular reac-

PLATE I

FIG. 1.—Case No. 61–36. Photomicrograph to show the normal metanephric tubules which differentiated in a graft of duck kidney tissue, the donor of which was a 13-day-old embryo, after having grown on the chorio-allantoic membrane of the chick for 8 days. × 300.

FIG. 2.—Case No. 63–29. Section of a graft of duck kidney tissue, the donor of which was a 19-day-old embryo, after having grown on the chorio-allantoic membrane of the chick for 8 days. Note the large area of normal metanephric tissue (*a*) and the relatively small area of necrotic tissue (*b*). × 100.

PLATE I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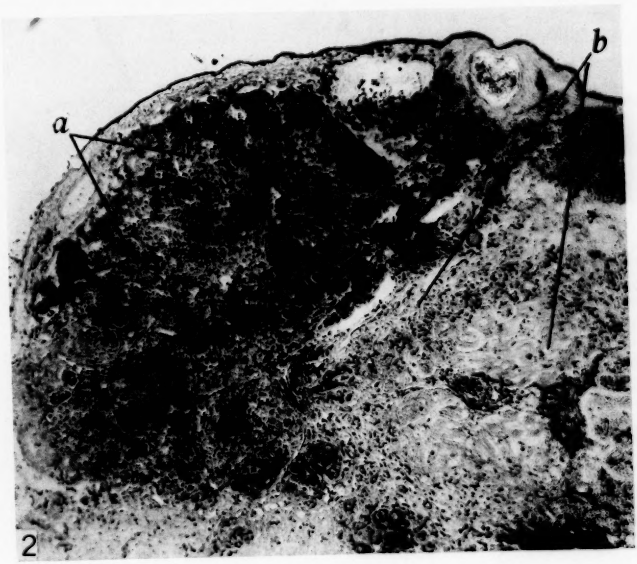

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tion (Plate I, Fig. 2). In Case No. 82-11 there is no evident necrosis. All of the grafts are completely included in the chorio-allantoic membrane of the host, and they are well vascularized. The normal metanephric tissue of the grafts has secreting and collecting tubules that are well formed, their cellular constituents displaying the usual characteristics. Mitotic figures are common in the cells, although perhaps not as common as in the younger tissue transplanted. Glomeruli are present and common.

In spite of the thorough vascularization of the grafts, all, with the exception already noted, have necrotic areas, usually of small extent, however. Both secreting and collecting tubules are included in the areas, and various phases of necrosis may be selected. Frequently the tubules remain intact and the cells are in their normal relationships. The nuclei, however, are not normal. Their staining reaction varies, and they are shrunken and distorted. In a few cases the nuclei seem to have disintegrated, and the resulting pieces have spread into the surrounding cytoplasm, which still remains characteristic for the most part, although in some instances it stains more deeply than normally.

In another selected phase of necrosis, the cells, although they are still in their normal relationships, thus preserving the shape of the tubules, have lost their nuclei entirely; or if the nuclei are present, they are without definite staining reaction. The cytoplasm has also been affected. It no longer stains, and often contains many vacuoles of varying sizes. The masses of necrotic tissue at this time are unstained and appear yellowish (Plate I, Fig. 2, *b*). In a more advanced stage of necrosis the cytoplasm of the cells is greatly distended because of the vacuoles, and as a result the tubules are distorted and broken down.

There exists at the same time as the necrosis just described, a definite cellular reaction in the tissue of the host. Fibroblasts have penetrated the spaces between the tubules, separating them into scattered groups. The tissue in these groups may or may not be normal, and it is difficult to determine whether the reaction is the cause or the result of the onset of necrosis.

The lymphocytes in these grafts are never so common as to be considered an infiltration as the term is usually used. In only one case,

No. 82-3, is there any indication that they might be slightly more numerous about the grafted tissue than one would expect to find in normal tissue. Granulocytes are not as common as they are in Series I; and are found, for the most part, in the interstitial tissue between the tubules of the grafted metanephric tissue.

Series III. Donors at 21–28 days of incubation.—There is very little difference between the histological structure of the grafts of this series and those of the preceding series except that there is considerably more necrotic tissue (Plate II, Fig. 3). The apparently normal kidney tubules, when present, are located in isolated groups in various parts of the graft, usually at the periphery. Case No. 66-37 is exceptional in that about one-half of the tissue present appears to be normal. Usually, little growth of the tissue has taken place and mitotic figures are scarce, being limited to the normal tubules. In all of the twenty-four cases in the series, necrosis is very evident.

The reactions of the host against the transplanted duck tissue are of two general types—either the graft is fully incorporated into the chorio-allantoic membrane of the host, and then becomes invaded or surrounded by connective tissue, or the transplanted tissue is sloughed off from the membrane and discharged into the adjacent space. This latter phenomenon, as indicated in Table I, occurs quite commonly in this series. Described in histological detail, the tubules appear compact and crowded. The cells usually have granular cytoplasm, and may be either with or without nuclei. For the most part, it is impossible to identify the secreting and collecting tubules; but in a few instances they have been recognized. Both are equally affected. The tissue of the graft itself is poorly vascularized. It is completely necrotic. In the immediate surroundings, however, are the large blood vessels of the host membrane.

The cellular reaction that accompanies sloughing is the same as that which effects a completely incorporated graft. Connective tissue replaces the necrotic tissue as it is sloughed off. In some cases, Nos. 65-4 and 65-5 as examples, the grafted tissue has been discharged from the membrane, and the region completely replaced with connective tissue. Whether the graft is incorporated or in the process of being sloughed off, lymphocytes are never common enough

to be considered an infiltration, and granulocytes are less common than they were in the grafts of the previous series.

Series IV. Donors at 29–38 days old (1–10 days extra-shell life).—The results of this series differ in no appreciable way from those of Series III. Case No. 50-15 (Plate II, Fig. 4) represents the typical condition found in the eleven cases of the series. There is some apparently normal tissue present which consists of isolated groups of tubules scattered about the periphery of the graft. Mitotic figures are rare and limited to the cells of the few normal tubules. The greater part of the transplanted tissue is necrotic. Case No. 53-33 is exceptional in that it is almost completely of normal tissue.

The reaction of the host is characteristic. In a few instances necrosis of the duck tissue is complete and sloughing has taken place. More often, the tissue is completely incorporated into the chick membrane, and connective tissue has infiltrated between the tubules or has encapsuled the transplant. Lymphocytes are never very common. Granulocytes, as is characteristic in all of the grafts made from older metanephric tissue, are either lacking entirely or are present in small numbers only.

Series V. Adult duck donors.—When kidney tissue is present in the grafts of this series, it is completely necrotic (Plate II, Fig. 5). In most cases the tubules still retain their form. They differ in no manner from the completely necrotic tubules of the foregoing series. The cellular reaction consists chiefly of connective tissue which encapsules or invades the transplanted tissue in the usual manner. Lymphocytes are wanting in numbers greater than would be expected in any other part of the chick membrane, and granulocytes are either lacking or sparse.

C. GRAFTS OF DUCK KIDNEY TISSUE MADE ON THE DUCK

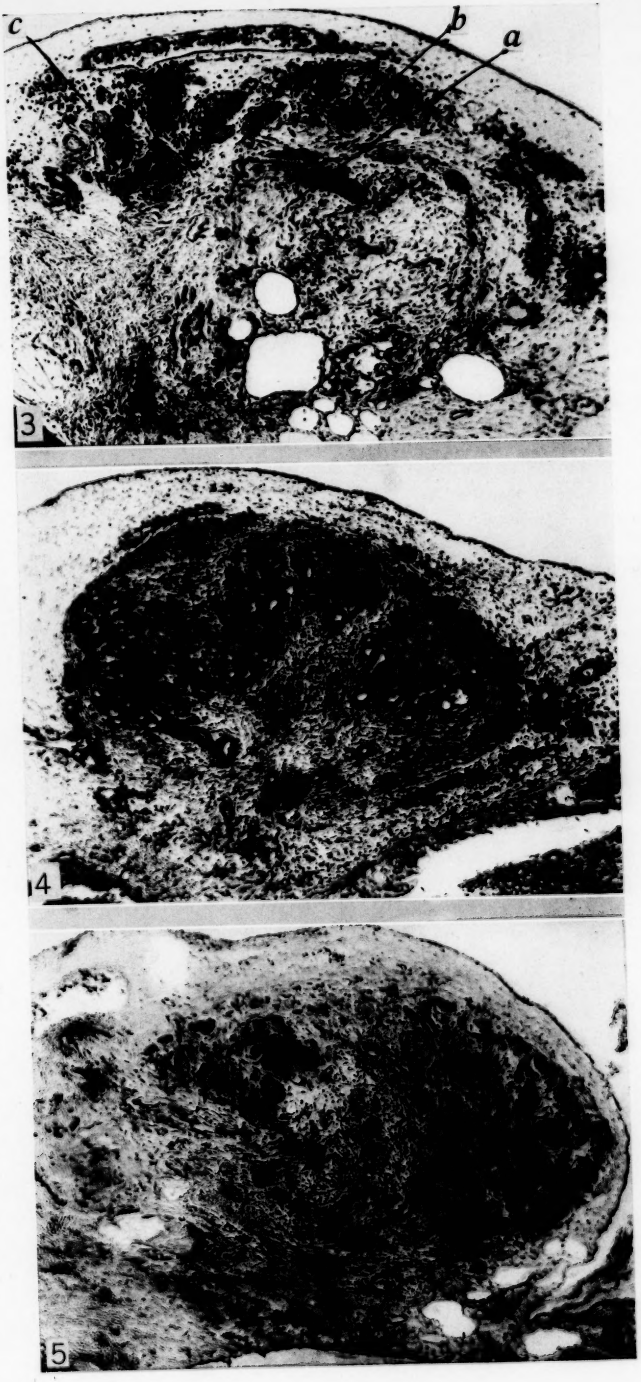
The results obtained from the transplantation of duck kidney tissue to the chorio-allantoic membrane of the duck are similar to those obtained from the transplantation of duck tissue to the chick. They are tabulated in Table II. The grafts resulting from tissue obtained from donors at 13 days of incubation show normal differentiation and considerable growth. Those obtained from transplants of kidney tissue from donors at 21 days of incubation show less growth, if

PLATE II

FIG. 3.—Case No. 67–8. Section of a graft of duck kidney tissue, the donor of which was a 28-day-old embryo, after having grown on the chorio-allantoic membrane of the chick for 8 days. Note the presence of connective tissue about the necrotic area (*a*) and necrotic tubules (*b*). Normal tubules (*c*) are few and scattered. × 70.

FIG. 4.—Case No. 50–15. Section of a graft of duck kidney tissue, the donor of which had been hatched for 1 week, after having grown on the chorio-allantoic membrane of the chick for 8 days. Note the large amount of connective tissue within the graft, and the persistence of the metanephric tubules, most of which are necrotic, but a few still in an apparently normal condition. × 70.

FIG. 5.—Case No. 45–30. Section of a graft of adult duck kidney tissue, after having grown on the chorio-allantoic membrane of the chick for 8 days, showing the extensive connective tissue infiltration and complete necrosis of persisting metanephric structures. × 70.


c
b
a
3
4
5

any, and varying amounts of normal and necrotic tissue, the extent of the latter increasing in the grafts resulting from transplants of kidney tissue from donors at 24 and 27 days of incubation. With the increasing amount of necrosis there is an accompanying increase in the amount of connective tissue. Lymphocytes are never abundant,

TABLE II

GRAFTS OF DUCK METANEPHRIC TISSUE ON CHORIO-ALLANTOIC MEMBRANE OF DUCKS

Case No.	Age of Host (Days)	Age of Donor (Days)	Time on Host (Days)	Total Age of Grafted Tissue (Days)	Size of Graft at the Time of Removal (Millimeters)	Normal Tissue	Necrotic Tissue	Cellular Reaction		
								Connective Tissue	Lymphocytes	Granulocytes
112-4	14	13	8	21	1.90×1.90×3.46	++++	−	−	−	+++
112-16	14	13	8	21	3.50×1.60×2.67	++++	−	−	−	+++
112-20	14	13	8	21	2.80×1.60×3.34	++++	−	−	−	+++
112-22	14	13	8	21	3.80×1.90×4.00	++++	−	−	−	+++
205-17	14	21	8	29	1.80×0.50×0.50	++	++	+	−	+
205-33	14	21	8	29	1.40×0.60×1.15	+++	+	−	−	+
205-34	14	21	8	29	1.10×0.60×0.75	++++	−	−	−	++
203-2	14	24	8	32	1.20×0.50×1.30	+	+++	+	+	+
203-9	14	24	8	32	1.30×1.70×1.28	+	+++	++	++	+
203-11	14	24	8	32	1.00×0.50×1.20	++	++	++	−	+
203-16	14	24	8	32	1.30×0.90×0.90	+	+++	++	−	−
202-2	14	27	8	35	1.00×0.50×0.96	+	+++	+++	−	−
202-7	14	27	8	35	1.10×0.70×1.18	+	+++	+++	−	+
202-9	14	27	8	35	1.00×0.80×1.09	+	+++	+++	+	−
202-13	14	27	8	35	0.80×0.50×0.66	++	++	++	−	−
202-14	14	27	8	35	0.70×0.90×0.96	+	+++	++	−	−
210-26	14	Adult	8	Adult	0.60×0.80×0.83	−	++++	+++	−	−
210-27	14	Adult	8	Adult	1.40×0.80×1.70	−	++++	+++	−	−
210-31	14	Adult	8	Adult	1.40×0.70×1.25	−	++++	+++	−	−

and granulocytes are common in the earlier stages but disappear in the later. Grafts of adult duck kidney tissue show complete necrosis. Although the grafts of duck kidney tissue made on the chorio-allantoic membrane of the duck do not differ a great deal from those made on the chick, there are slight indications that more normal, and less necrotic and connective, tissue is present. These differences, however, are so slight as not to be apparent upon comparing Tables I and II.

DISCUSSION

The results of the experiments show that the capacity of duck metanephros to grow on the chorio-allantoic membrane of either the

duck or the chick is correlated with the degree of differentiation of the donor tissue at the time of transplantation. This is demonstrated by the fact that metanephric tissue from an embryonic duck of 13 days' incubation will grow and differentiate in an apparently normal manner when transplanted to the membrane of either of these hosts. Metanephric tissue from successively older donors, grafted in a like manner, shows successively lesser amounts of normal and greater amounts of necrotic tissue. As has already been noted, the undifferentiated tissue of the metanephros of a normally developed embryo greatly exceeds the differentiated at the thirteenth day of incubation, the earliest stage transplanted. Any transplant made at this stage contains mostly undifferentiated tissue which differentiates into normal metanephric structures. But transplants from a 19- or 21-day-old donor, for example, contain varying amounts of differentiated and undifferentiated tissue, depending on the region of the kidney from which the tissue for transplantation is taken. The amounts of normal and necrotic tissue in grafts resulting from such transplants varies from relatively large areas of normal and small areas of necrotic to small areas of normal and large areas of necrotic. Since in the normal development of the kidney, differentiation is not complete until after hatching, transplants made from later stages, although consisting mostly of differentiated tissue, may possibly contain some which is still undifferentiated. After remaining on the host membrane, whether duck or chick, the resulting grafts are, in most instances, completely necrotic, with varying degrees of connective tissue infiltration and replacement. If areas of normal tissue are present, they are small and scattered, and can be accounted for by the still undifferentiated nephrogenous tissue present at the time of transplantation. In the adult kidney, differentiation is complete, so that any transplant will consist entirely of fully differentiated tissue. After 8 days on either host, it becomes necrotic and even replaced by connective tissue.

The occurrence of necrosis and connective tissue in the grafts after they have reached a certain degree of differentiation indicates that the donor tissue has acquired certain specific properties, the nature of which has not as yet been determined. The importance of the tissue, individual, and species differentials in determining the trans-

plantation capacities of various tissues has been made apparent by the work of Loeb. Since undifferentiated nephrogenous tissue will grow and differentiate normally in chorio-allantoic grafts while differentiated metanephric tissue will not grow, it is evident that these differentials are lacking in the embryonic tissue and that they develop simultaneously during the process of differentiation. They are, therefore, difficult to separate experimentally. The presence of connective tissue and necrosis in the grafts of differentiated metanephric tissue made to the chorio-allantoic membrane of the duck (homoplastic transplants) suggests that the tissue and individual differentials are the responsible factors. Obviously, these same differentials are present in the differentiated metanephric tissue transplanted to the chick (heteroplastic transplants), and are, no doubt, responsible to a certain extent for the reactions appearing in these grafts. However, since these transplants involve a host of another species, the species differential must also be present and operative at the same time. Although the reactions are similar in the homoplastic and heteroplastic grafts, they are slightly more intense in the latter; and this difference in intensity, even though very slight, may be important as an expression of the species differential, since the work of Loeb and Wright (1927) has shown that in guinea pigs the degree of relationship between the host and donor determines the intensity of the reaction.

Another factor of seeming importance in determining the capacity of duck kidney tissue to grow on the chorio-allantoic membrane is the ability of the transplanted tissue to carry on its secretory function. Willier (unpublished data) found that homoplastic grafts of chick gonad will grow on the chorio-allantoic membrane without signs of degeneration, while Holton (1929) points out that the degeneration of chick liver in homoplastic grafts is correlated with a functional change in the liver cells. No evidence, however, is available, either from the experiments reported in this paper or from the literature, that the kidney tubules are functional at an age which approximates the time of appearance of necrosis in the grafts. In the differentiation of chick metanephros in homoplastic grafts, Atterbury (1923) stated that the tissue probably reached a functional stage because the staining reaction of the tubules changed as they

differentiated into secretory tubules. This same change in the staining reaction was noted in the tubules of both the homoplastic and heteroplastic transplants of duck kidney tissue, but, other than showing a change in the nature of the cytoplasm of the cells, is hardly to be considered as adequate evidence of an actual secreting function. Furthermore, if the degeneration of the kidney is correlated with its secretory function, then the secreting tubules should degenerate first. Nothing in the grafts indicates that this is true, for both the secreting and collecting tubules seem to be equally affected. This point, however, can bear further investigation.

Since these results show that the capacity of the metanephric tissue of the duck to grow on the chorio-allantoic membrane of the chick is correlated with the degree of differentiation of the donor tissue at the time of transplantation, it seems logical to assume that different tissues will grow in chorio-allantoic grafts for different lengths of time, for it is well known that, in the development of the embryo, they neither differentiate at the same rate nor become fully differentiated at the same time. Whether the results of Hiraiwa (1927) can be interpreted in the light of this assumption is not certain, but highly suggestive. He transplanted sectioned rat embryos which were about 11 days old to the chorio-allantoic membrane of the chick. After growing 8 or 9 days on the chick membrane, little or no nervous tissue was found in the grafts, while such structures as epidermis and cartilage were found. The fact that nervous tissue was not found suggests that, at the time of transplantation, it had already differentiated to that stage when it would no longer grow on the host membrane. On the other hand, epidermis, in which the *stratum germinativum* retains an active embryonic condition through most of the life of the individual, showed a high degree of persistence. To what extent the differential factors and functional ability were responsible for the results cannot be determined from the descriptions available. However, Hiraiwa and Willier (1927), in a preliminary report, mentioned the possibility of another factor, namely, differential survival, which is based on the capacity of tissues to resist the reactions of the host because of their structural peculiarities.

The question also arises as to whether the reaction occurring against the differentiated metanephric tissue transplanted to either

the chick or the duck is dependent in any way on the presence of specific properties in the host. Although the results of this study do not show that such properties exist at the time the grafts are removed, still there is the suggestion that, if related to the degree of differentiation, they should become more and more established as differentiation proceeds. It seems, therefore, that they should be fairly well established before hatching, because at this time the embryo is already a rather highly differentiated individual. This is, in fact, what the results of Murphy (1914*a*, 1914*b*, 1926) indicated. He found that a species specificity developed in the host embryo sometime after the eighteenth day of incubation, because embryonic and sarcomatous tissue of the rat grafted to the chorio-allantoic membrane of the chick grew until the eighteenth day but not thereafter. He also demonstrated that it is definitely present in the chick at the time of hatching, since embryonic rat tissue grafted to the newly hatched chick did not grow. From the results of his various experiments, he concluded (1914 *b*, pp. 551) that "the chick embryo offers suitable conditions for the growth of implanted tissue, whether these be embryonic or adult, of the same species or a foreign one," and that "the chick at about the time of hatching develops a mechanism against the tissue of a foreign species." His evidence concerning the reaction against grafts removed after the eighteenth day of incubation of the host is open to question because the chick usually hatches on the twenty-first day, and between the eighteenth and twenty-first days there are changes taking place in the chorio-allantoic membrane which makes it unsuitable for grafts. At least by the twentieth day the chorio-allantoic circulation is becoming affected, as is evidenced by the more viscid nature of the membrane; and this reflects on its value as a medium for growth of any tissue after this time. Furthermore, these experiments demonstrate conclusively that duck kidney tissue will not grow on the chorio-allantoic membrane of the chick after it has reached a certain stage in the process of differentiation even though the host is only seventeen days old. More recent experiments, still in the process of analysis, show necrotic tissue and an accompanying connective tissue proliferation in duck kidney tissue growing on chick hosts which are only at 12 days of incubation at the time the grafts are removed. This suggests that either the reaction

against the foreign tissue can take place independently of the degree of differentiation of the host or that the host acquires properties of a specific nature capable of producing the reaction at an earlier stage than that shown by Murphy.

SUMMARY

1. This paper is concerned with the growth of metanephric tissue from variously aged ducks transplanted to the chorio-allantoic membrane of the chick and duck.

2. The results show that undifferentiated nephrogenous tissue will grow on the chorio-allantoic membrane of either the chick or the duck, and differentiate into apparently normal metanephric structures. Fully differentiated metanephric tissue, however, will not grow on either type of host, but becomes necrotic; and the grafts become infiltrated with connective tissue. The amount of necrosis and connective tissue is roughly proportional to the amount of fully differentiated kidney tissue present in the transplant at the time of implantation.

3. It is concluded from these experiments that the capacity of duck kidney tissue to grow in chorio-allantoic grafts is dependent on the degree of differentiation of the donor tissue at the time of transplantation.

4. The factors responsible for the failure of the duck kidney to grow in chorio-allantoic grafts have not, as yet, been determined. It is suggested that the reaction against the transplants made to the duck is due to the development of the tissue and individual differentials in the donor tissue. The presence of a slightly more intense reaction against the duck tissue transplanted to the chick indicates that, in addition to the tissue and individual differentials, the species differential develops at the same time and is partly responsible for the results in the heteroplastic grafts. The ability of transplanted tissue to carry on its secretory function in chorio-allantoic grafts is also considered as a factor in determining its capacity to grow.

5. The question as to whether the host must develop a specificity before it will react against the foreign-tissue transplant is considered. This study does not demonstrate that the host is specific at the time the grafts are removed; but if specificity and differentiation are re-

lated, then it seems likely that the host is specific, for, by this time, it is already a highly differentiated individual. The evidence thus far obtained indicates either that the reaction can take place independently of the age of the host or that the host becomes specific at a much earlier age than has previously been supposed.

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HISTO-PHYSIOLOGICAL STUDY OF THE GLANDS OF INTERNAL SECRETION AND THEIR EFFECT ON OSSIFICATION IN THE CHONDRODYSTROPHIC AND "STICKY" CHICKEN EMBRYO

(One plate)

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INTRODUCTION

DIETERLE (1906) and others have described congenital athyroid in the imperfectly ossified fetus. Landauer (1927, 1929*a*, 1929*b*) described a morphological alteration in the bones and in the thyroids, thymii, parathyroids, and pineal body of the chondrodystrophic chicken embryo. The descriptions given by early workers suggest that the malformation of the embryo might be due to deviation from the normal activities of the endocrine organs. Therefore, a study was undertaken as a preliminary survey on the possible relation between the functional distortion of the endocrine organs and the malformation of the embryo.

The study includes a morphological observation of the thyroid, parathyroid, thymus, and adrenal glands; a chemical study of the iodine content of the thyroid glands; the epinephrin content of the adrenal glands; and an analysis of the chemical composition of the bones of the chondrodystrophic, sticky, and the normal[1] chicks. The results obtained from the foregoing animals were compared with those of Leghorn chicks of the same age.

The eggs of the deformed chicks were provided by the United States Department of Agriculture, Bureau of Animal Industry, Washington, D.C., through the courtesy of Dr. F. A. Woods, to whom the author is indebted. Detailed information concerning the family history and other conditions of these chicks may be obtained from the United States Department of Agriculture, Bureau of Ani-

[1] The term "normal chicks" is used to indicate those which were obtained from the eggs of the same lot which provided the deformed chicks.

mal Industry, Washington, D.C. The author is also indebted to Dr. J. H. Bodine and the Zoölogy Department of the State University of Iowa, for courtesies shown him and for the making possible of these investigations.

PROCEDURE

The eggs were incubated; and the thyroid, parathyroid, adrenal, and thymus glands were removed from the embryo at 17 days of incubation. In order to make the morphological observations, the glands were fixed in Bouin's fluid and 6 μ sections were mounted and stained with eosin and haematoxylin.

For the chemical determination of the active principle of the thyroid gland, the method of Leitch and Henderson (1926) was used. The epinephrin content of the adrenal glands was determined according to the technique of Folin, Cannon, and Denis (1913) and Sun (1929, 1931). For the study of the chemical compositions of the bones, the hind limbs, as representative of the whole skeletal system, were used and were treated according to the method of Hammett (1924). By this method, the values of the organic matter and water content could be obtained.

The weighed ash of the bones, after having been dissolved in 1 milliliter of approximately normal hydrochloric acid, was transferred into a 50-milliliter volumetric flask and diluted to the mark with water. The amount of ash solution used for the analysis was determined by its concentration. In general, it was about 1 or 2 milliliters for calcium and from 5 to 40 milliliters for phosphorus. The method of Kramer and Tisdall (1921), and of Tisdall (1923) was used for analyzing the calcium; and that of Fiske and Subbarow (1925), for phosphorus. All figures were computed into percentage of the tissue.

Twenty-one normal, eight chondrodystrophic, and eleven sticky chicks were used. Leghorn chicks of the same age were used in all cases for comparison.

OBSERVATIONS

The chondrodystrophic and sticky chicks were recognized by a deformed head and feeble hind limbs, the bones of which were small and soft as compared with those of normals of the same age. Also, the sticky chicks show an edematous condition in the subcutaneous layers.

The thymus, thyroid, and adrenal glands in the deformed chicks were found to be reduced in size; but the parathyroids were enlarged. All the glands showed a pathological condition. The degree of deformity of glands, bones, and other features varied with the individual.

No attempt was made to record the weights of the parathyroid and thymus glands, since the morphological differences in the glands of the deformed chicks were markedly contrasted to the normals. Weights were recorded only on the thyroids and adrenals, because in them the active principle seemed to be present.

The weights of the thyroid glands per pair were 18.5×10^{-4} mg. for the normal, 9.5×10^{-4} mg. for the chondrodystrophic, and 5.5×10^{-4} mg. for the sticky chicks, while that of Leghorn chicks was 22.6×10^{-4} mg. The iodine content of the thyroid glands was 0.65 per cent for the normal, 0.65 per cent for the Leghorn chicks, 0.169 per cent for the chondrodystrophic, and was not detectable in the sticky chicks. The iodine percentage in the glands of the normals was not different from that of the Leghorn, but in the chondrodystrophic and sticky chicks it was practically negligible.

From the morphological examination, no striking changes were found in the thyroid, except that the colloid substance in the vesicles showed, more or less, a heterogeneous condition. Landauer (1929*a*, 1929*b*) described the thyroid in the chondrodystrophic chicken embryo and showed that the vesicles are less in number and smaller in size and that there is a larger amount of epithelial tissue between the vesicles as compared with that of normals. It is very like the condition in the early stage of the Leghorn chicks.

In general, the thymus glands of the deformed chicks showed slight histological difference from those of normals; but in extreme cases, a very little cell mass, surrounded by an abundance of connective tissue, was found. The cortex and medulla of the glands were undifferentiated, and no Hassall's corpuscles were found. The glands appeared to be in an undeveloped or degenerated condition.

Superficial examination of the parathyroid showed it to be enlarged in size. No attempt was made to record the weight, since the glands are small and the probable error would have been great. The sections showed that the cells at the peripheral region were elongated

and failed to take the stain. Normal tissue appeared at the center of the section, retaining a cordlike structure. For the purpose of comparison, the sections of both the normal and deformed glands are represented by Figures A and B in Plate I.

No striking histological changes associated with the change in size of the adrenal gland could be noticed. The gland weight was 4.47 mg. for the Leghorn, 3.05 mg. for the normals, 2.6 mg. for the chondrodystrophic, and 2.9 mg. for the sticky chicks. By the chemical test, the epinephrin concentration per gram of gland tissue was found to be 2.44 mg. for the normal chicks, 2.28 mg. for the chondrodystrophic, 2.31 mg. for the sticky chicks, while that of the Leghorns was 1.78 mg. The total amount of epinephrin in the glands per pair was 5.92×10^{-3} mg. for the chondrodystrophic, 6.7×10^{-3} mg. for the sticky, and 7.05×10^{-3} mg. for the normals, while that of the Leghorn was 7.9×10^{-3} mg. The low values hold for the smaller glands.

Of significance is the fact that the foregoing data indicate that the growth of the glands was retarded to a greater degree in the cortex than in the medulla. This is probably due to the fact that the cortex is more susceptible to the unfavorable condition.

Now turning to the bone analysis, the purpose of this work was to determine the relative amounts of the chemical constituents which participated in the ossification of the embryo as a result of the distortion of the glands of internal secretion. The treatment and technique were stated above.

Since a morphological description of the bones of deformed chicks was given by Landauer (1929*a*, 1929*b*), it is not necessary to repeat it here. In making the chemical analysis, I first considered the calcium, phosphorus, and the organic matter, since they should reach a definite relative proportion at a certain stage of the development of the embryo. Other substances present in the bones are, of course, significant, but are present only in small amounts. The numerical values of the different elements are given in Table I.

The results showed that the deformed chicks possess a higher percentage of organic matter and a lower percentage of calcium and phosphorus. Likewise there is a lower percentage of water content. The range of these substances is greater in the deformed chicks than in the normals. The chemical composition of the bone is chiefly cal-

cium phosphate and other calcium salts, the ratio between the total calcium and the total phosphorus of well-developed bones being 1:2.1 at 17 days of incubation. But in the deformed chicks it was 1:2.65. It is significant that the deformed chicks showed deficiency not only in calcium but also in phosphorus in a greater proportional degree. The lower percentage of water content is probably due to the small capacity of the marrows or other cavities in the bones, which are not properly formed.

TABLE I

THE CHEMICAL ELEMENTS OF THE BONES

Type of Experimental Animal	Water Content (Percentage of Fresh Weight)	Organic Matter (Percentage of Dry Weight)	Phosphorus (Percentage of Dry Weight)	Calcium (Percentage of Dry Weight)	Other Substance (Percentage of Dry Weight)	Ratio of Phosphorus to Calcium
Normal:						
Average.....	71.8	61.45	6.71	14.9	16.94	1:2.2
Range.....	60–73.8	54.03–76.5	4.8–8.3	12.98–17.14		
Leghorn:						
Average.....	76.5	59.8	7.257	15.13	17.813	1:2.086
Range.....	74.8–77.5	34.4–61.58	7.017–7.71	14.41–15.94		
Chondrodystrophic:						
Average.....	67.05	60.5	8.37	13.04	17.09	2 specimens only
Range.....	61.3–72.8	60.5	5.2–11.54	12.69–13.4		
Sticky:						
Average.....	68.7	75.92	4.62	12.278	7.176	1:2.65
Range.....	41–80	60–85.8	1.8–7.43	6.71–16.5		

DISCUSSION

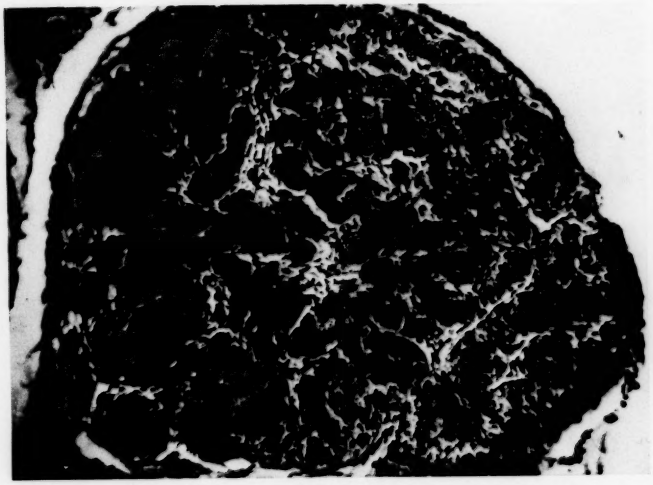
The external features and the morphological deviation of the bones and the endocrine organs of the chondrodystrophic chicken embryo, as described and discussed by Landauer (1927, 1929*a*, 1929*b*) and others, varied in each individual according to the degree or kind of deformity, and therefore need not be compared with the results obtained here. The discussion will therefore be confined to the present data.

From the morphological point of view, the deformed chicken embryo, as compared with the normals of the same age, is much smaller in size. The head is large in proportion to the size of the body, and the limbs are feeble. An extreme case showed quite a marked edema-

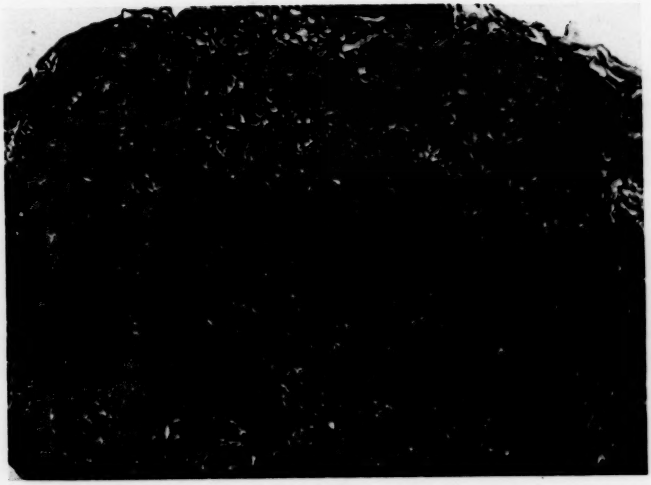
PLATE I

FIGS. A AND B.—Sections showing the difference in structure of the parathyroid glands of normal and deformed chicks, respectively.

PLATE I



A



B

tous condition in the subcutaneous layer. On autopsy it was found that the endocrine organs were pathological.

From the physiological point of view, it was found that in the deformed chicken embryo the iodine content of the thyroid glands was undetectable and that the metabolism of the chemical elements of the bone was distorted.

Metabolism has been long believed to be controlled by the endocrine organs, each organ furnishing a specific function toward the balance of the living mechanism. This applies probably to the postnatal life. In the embryonic life of chicks, the organs of the incretory system, besides the thyroid and adrenal glands, are mostly inactive. Furthermore, concerning the rôle of the adrenal gland, both cortex and medulla, from the metabolic point of view, it is in the catabolic, rather than anabolic, phase. The regulation of metabolism in the embryonic life of the chick may then probably be attributed to the thyroid gland only. The evidence of this was obtained from the fact that in deformed chicks, the active principle of the thyroid glands was practically negligible, while the adrenal glands and their contents changed in less degree. Nor is there any evidence to show that the other glands exert any direct influence on metabolic activities in embryonic life. I am inclined to the idea of Dieterle (1906) that the thyroid gland is essential to ossification in the fetus. Thus the deficiency of thyroid secretion is the main cause of the malformation of the chicken embryo and is followed by changes in the morphology and physiology of the organs and tissues as a whole.

CONCLUSIONS AND SUMMARY

The thyroid, parathyroid, adrenal, and thymus glands were examined microscopically at 17 days of incubation; and the iodine content of the thyroid glands, the epinephrin content of the adrenal glands, and the bone compositions were chemically determined. The results obtained from the deformed chicken embryos were compared with those found in both the Leghorns and the normals of the same lot of eggs as furnished the deformed chicks. The fact that deficiency of thyroid secretion is followed by disturbance in the metabolism was established. The significant results were as follows. In deformed animals—

1. The thyroid, adrenal, and thymus glands were found to be decreased in size. The parathyroid glands were found to be enlarged. All of the glands showed a pathological condition.

2. No striking changes in the microscopic structure of thyroid and adrenal glands associated with the changes of gland size could be detected. The thymus glands showed a degenerated or undeveloped condition. In sections of the parathyroid, the cells of the peripheral region were found to be elongated, and the cytoplasm failed to take the stain.

3. The iodine content of the thyroid glands was undetectable. The epinephrin determination showed that the growth of adrenal cortex was retarded in a greater degree than in the medulla.

4. The metabolism of calcium, phosphorus, and organic matter of the deformed chicken bones was distorted. The degree of deficiency of phosphorus is greater than that of calcium. The percentage of water content of the bones of deformed chicks is lower than that of normals.

From these findings, a conclusion will be drawn that the malformation of the chicken embryo is due to a deficiency of thyroid secretion which distorts the metabolism of the animal body as a whole.

A conclusion is drawn that the malformation of the chicken embryo is due to a deficiency of thyroid secretion which distorts the metabolism of the animal body as a whole.

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HISTO-PHYSIOGENESIS OF THE GLANDS OF INTERNAL SECRETION—THYROID, ADRENAL, PARATHYROID, AND THYMUS—OF THE CHICKEN EMBRYO

(Six figures)

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INTRODUCTION

IT IS of physiologic as well as morphologic importance to understand the activities of the glands of internal secretion during the process of development of the embryo, since it has been believed that the endocrine organs are essential for the growth of the animal. An account of the morphogenesis of the thyroid glands in the bird was first given by Müller (1871) and later by Bradway (1929); and of the adrenal glands, by Rabl (1891) and Hays (1914). The physiological importance of both glands in the embryo has, however, remained practically untouched.

By means of the staining reaction of the colloid substance in the thyroid, Bradway (1929) assumed that the glandular function is probably commenced after 12 days of incubation. Uhlenhuth and Ebeling (1925) demonstrated that the metamorphosis of the larvae of *Amblystoma tigrinum* may be accelerated by feeding with the thyroid of an 18-day chicken embryo. Hogben and Crew (1923) stated that the active principle of the adrenal glands increases after 15 days of incubation. A more detailed study than this, however, seems to be desirable. The present paper includes a study of the physiogenesis as well as the morphogenesis of the thyroid and adrenal glands and also of the parathyroid and thymus, with special reference to the later stages of the development of the chick.

MATERIAL AND TECHNIQUE

Freshly laid white Leghorn eggs were used. The thyroid, parathyroid, adrenal, and thymus glands were removed from well-developed chicks at 24-hour intervals from approximately 9 days (216 hours) of incubation until hatching time.

In order to make the morphological observations, the glands were fixed in Bouin's fluid, and 6 μ sections were mounted and stained with eosin and haematoxylin. For the iodine determination, a number of pairs of weighed thyroid glands of the same age as those used for the morphological observation were subjected to the iodine test according to the method of Leitch and Henderson (1926) with the exception that the sodium thiosulphate was of N/5,000 instead of N/500 and was standardized against potassium dichromate before using. By this method, it is possible to detect a minimum figure of iodine of 1/200 mg. if care is taken.

A number of pairs of weighed adrenal glands from the same animals as supplied the thyroid glands for the iodine test were used for the epinephrin determination according to the method of Folin, Cannon, and Denis (1913). The method for the tissue extract preparation and further treatments were as of Sun (1929). The uric acid reagent was prepared according to Folin and Marenzi (1929). Standard uric acid was of 0.1 mg. per milliliter, of which the color intensity is proportional to the concentration of uric acid used. The value of the readings obtained from the colorimeter was reduced to milligrams per gram of gland tissue.

During the course of preparation, the adrenal glands, after removal from the embryo, were kept in 0.5 milliliter of N/10 hydrochloric acid, which was pipetted into a centrifuge tube. The thyroid glands likewise were put into a small vial and treated with 0.5 milliliter of 20 per cent potassium hydroxide, and the whole was closed in a weighing bottle. Both glands were placed in a cooling bath to prevent evaporation and to avoid chemical processes occurring in the tissue.

The environment and treatments were the same for all, regardless of the sex, since the hormones of the gonads are believed not to be active before puberty. Only pure-line white Leghorn eggs were used, in order that the hereditary constitution might not be an interfering factor.

OBSERVATIONS AND RESULTS

The observations included a morphological study of the thyroid, parathyroid, adrenal, and thymus glands and a chemical study of the thyroids and adrenals from 9½ days until the hatching period. No

attempt was made to determine the cytological significance or to trace the origin of the glands, since they have already been well described in various groups of animals.

The thyroid glands are located at the junction of the subclavian and carotid arteries along the internal jugular vein. At six days of incubation the gland is found to have a cordlike structure. The vesicles in the tissue first appear at 9 days (216 hours) of incubation, and at that time the epithelial cells of the vesicles are of the cuboidal

TABLE I

THE THYROID GLANDS—THEIR GROWTH AND THEIR IODINE CONTENTS

Age (Days)	No. of Embryos	Gland Weight per Pair (Milligrams)	Iodine in Fresh Tissue (Percentage)	Total Iodine in Gland (Milligrams 10^{-3})
9–9½	20	0.3	.....	.....
10–10½	30	.38	0.23	0.2345
11½	20	.605	.409	.28504
12½	20	.9155	.3690	.3696
13–13½	30	0.95	.31758	.2569
14–14½	30	1.13	.338	.476
15–15½	40	1.13	.4546	0.486
16–16½	40	1.54	.6516	1.095
17–17½	33	2.37	.96	2.2752
18	20	2.2194	.6629	1.4712
19	16	3.245	.55	1.78475
20	10	3.41	0.92	3.137
20¼	2	6.2	1.31	8.122 (after hatched 6 hours)

type. Thereafter there is an increase in the amount of glandular tissue and in the number and size of the vesicles. At 15 days of incubation the glands appear practically the same as those of the adult animal, and the epithelial cells of the vesicles are more or less flattened.

The iodine determination was performed as soon as the vesicles appeared. It was found that the iodine contents present in the glands increased with age of incubation. The increase of absolute value from 9 days to the end of incubation was about 13 times, and to the period of 6 hours after hatching was about 34 times. The percentage increase was about 4 times for the former period and 6 times for the latter, while the gland growth was about 10 times for the former and about 20 times for the latter. This determines the growth relation

between the gland tissue and its active principle. The growth curve of the glands and the numerical values of the iodine contents are given in Table I and Figures 1–3. At the eighteenth day of incubation, the values of both percentage and absolute iodine content seem

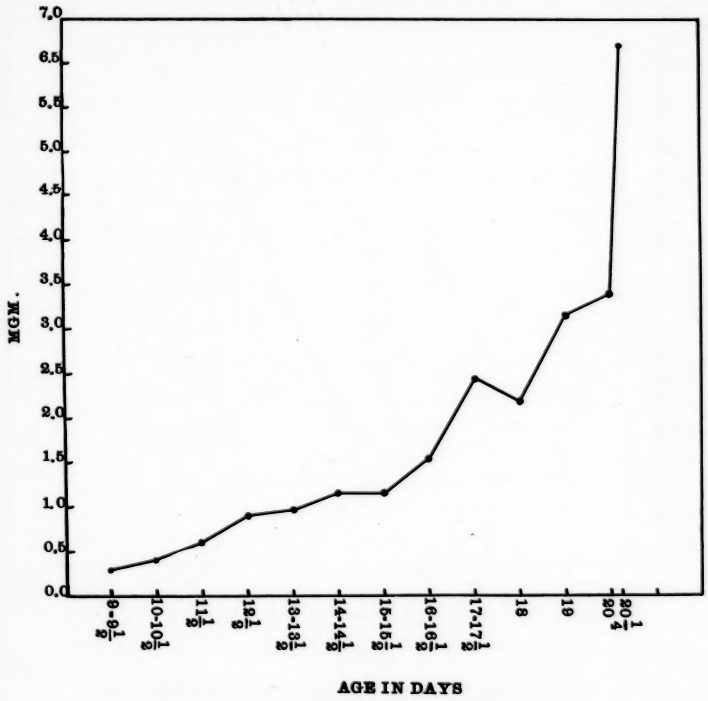

FIG. 1.—Weight per pair of thyroid glands

to drop off. The reason for this is uncertain. But even though the variability is great, a general tendency shows that the iodine content definitely increases. Bradway (1929) stated that the vesicles first appeared at 11 days of incubation; and at 12 days the colloid substance in the vesicles showed a chromophilic reaction, by which it was assumed that the thyroids had begun to function. Thus the morphological point of view does not quite agree with the chemical determination.

The parathyroid glands are usually associated with, and posterior to, the thyroid glands. At the eighth day of incubation the gland tissue is cordlike in structure, somewhat similar to the early stage of the thyroid. It differs from the thyroid in that the glandular tissue

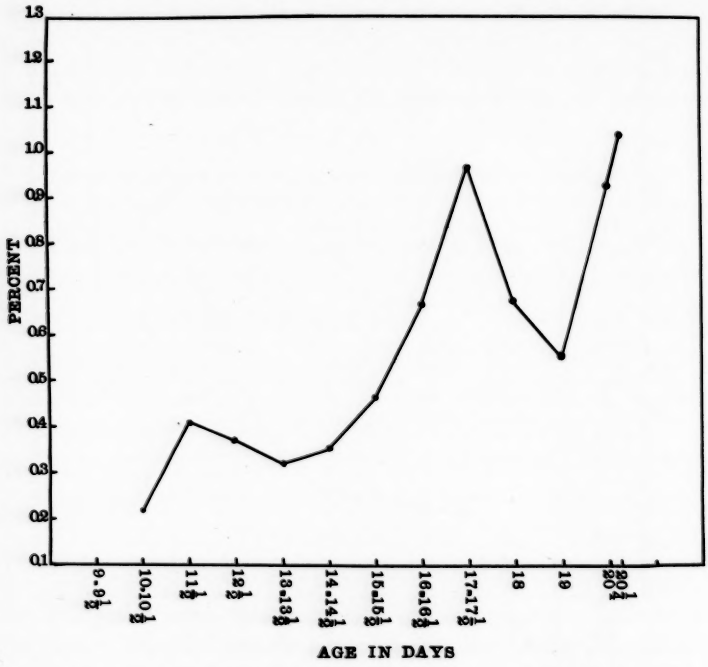

FIG. 2.—Percentage of iodine content per pair of fresh thyroid glands

forms a relatively thicker cord and no vesicles are present. At the last two or three days of incubation, the gland becomes very similar in appearance to the adult gland, but it still retains its cordlike structure and the cell groups are seen to be separated by connective tissue. Therefore, since these characteristics are retained throughout practically the entire period of embryonic life, it is rather doubtful that the gland has any function in the embryo.

Adrenal glands of $9\frac{1}{2}$ days of incubation consist of merely a soft, loose, and whitish cell mass located at the ventral side of the kidney

and anterior to the gonads. At 12 days of incubation, the gland tissue becomes more compact in structure, resembling very closely the adult gland. The morphogenesis of the glands need not be repeated

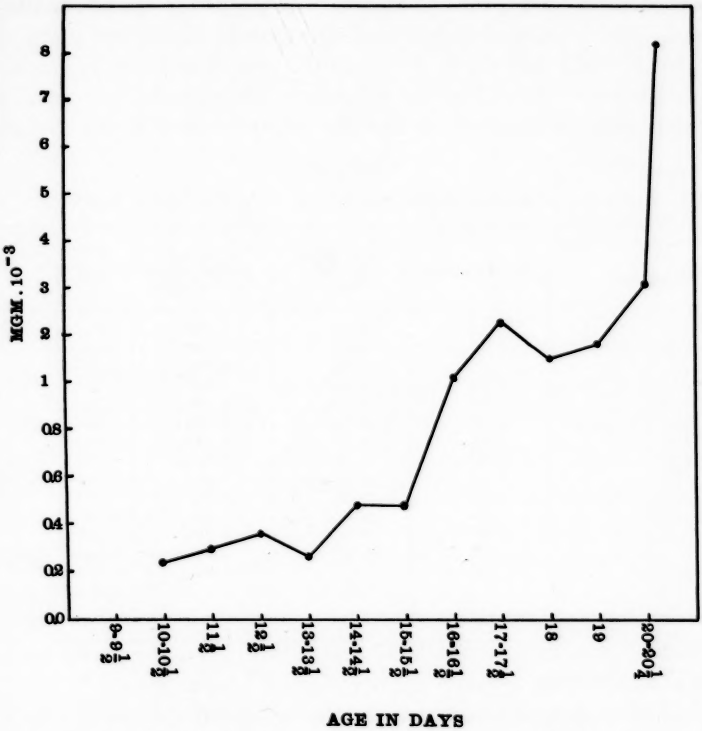

FIG. 3.—Total iodine content per pair of thyroid glands

here, since a good account has been given by Rabl (1891) and Hays (1914).

The epinephrin contents of the glands were determined from 9½ days of incubation and were found invariably to increase in amount with the age of the embryo. The increase of absolute amount of the epinephrin contents from 9½ days to the end of incubation was about 26 times, and to the period of 6 hours after hatching was 45 times. The figure in milligrams per gram of gland tissue was about 4 times

for the former period and 6 times for the latter. The growth of the gland tissue was about 6–7 times for both periods. This determines the degree and nature of the growth of both the cortical and medullary tissue in the glands and also the amount of the epinephrin contents. The numerical values and the growth curves are given in Table II and Figures 4–6. At about the seventeenth day of incubation the curve shows that the epinephrin concentration per gram of gland tissue dropped off, as did the iodine content of the thyroid

TABLE II

THE ADRENAL GLANDS—THEIR GROWTH AND THEIR EPINEPHRIN CONTENT

Age (Days)	No. of Embryos	Gland Weight per Pair (Milligrams)	Epinephrin (Milligrams per Gram of Tissue)	Absolute Epinephrin (Milligrams per Pair 10^{-3})
$9\frac{1}{2}$	10	0.99	0.661	0.06544
$10\frac{1}{2}$	10	1.16	1.025	.119016
$11\frac{1}{2}$	20	1.34	0.825	.108212
$12\frac{1}{2}$	20	1.42	1.2415	.1757
$13–13\frac{1}{2}$	30	2.185	1.5245	.33297
$14–14\frac{1}{2}$	30	2.635	1.5831	.41817
$15–15\frac{1}{2}$	25	3.18	1.6593	.595733
$16–16\frac{1}{2}$	40	3.60	2.0055	.71671
$17–17\frac{1}{2}$	35	4.47	1.79	0.798
18	14	5.06	2.40	1.214
19	16	5.454	2.53	1.38
20	10	6.634	2.376	1.57623
$20\frac{1}{4}$	2	7.55	3.84	2.8992 (after hatched 6 hours)

glands. It was suspected that the reason for this was a possible functional relationship between the thyroid and adrenal glands.

Before 15 days of incubation the thymus gland appears as only a group of undifferentiated cell masses linked together by connective tissue. The demarcation into cortex and medulla and Hassall's corpuscles does not definitely show until 15 days of incubation. From the morphological point of view, the function of the thymus glands, if any, probably commences after 15 days of incubation.

DISCUSSION

The endocrine organs compose a system of co-ordination or subordination in maintaining the balance of the living mechanism, including the growth and behavior of the animal. This applies to the

adult animal after puberty. No reference to the embryonic endocrine system in birds could be found in the literature except a paper by Uhlenhuth and Ebeling (1925) dealing with the acceleration of metamorphosis of the larvae of the salamander by feeding with the

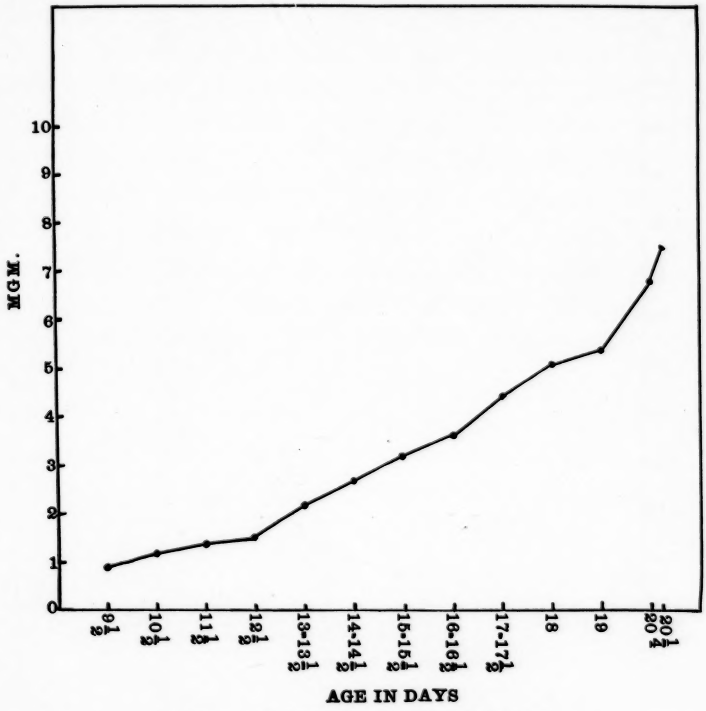

FIG. 4.—Weight per pair of adrenal glands

thyroid of an 18-day chicken embryo. Bradway (1929), by means of the chromophilic reaction of the colloid substance in the vesicles, assumed that the function of the thyroid of the chicken embryo begins at 12 days of incubation.

It is generally believed, however, that a morphological demonstration is not an adequate means of interpreting the physiological significance and that a feeding experiment is too arbitrary and involves too many conditional factors.

In studies on mammals, Kull (1926), by the histological method, stated that the function of the thyroid of rats began from the last three or four days of embryonic life. Fenger (1912) found, however,

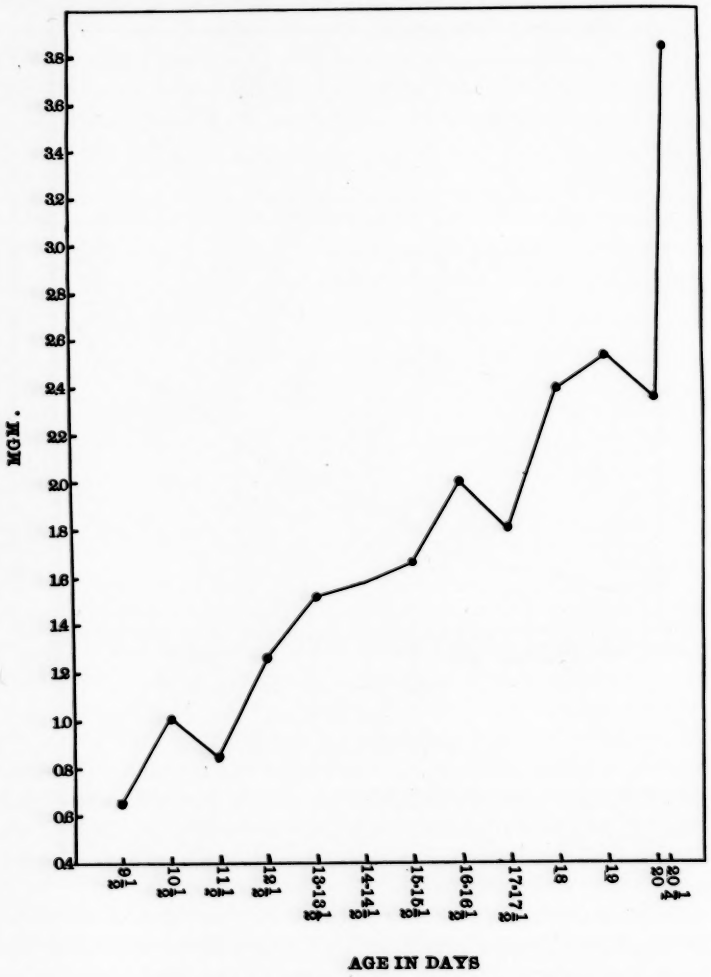

FIG. 5.—Epinephrin content per gram of gland tissue

that the thyroid of mammals contains no iodine before birth. This controversy is obviously due to phylogenetic differences and need not be discussed here.

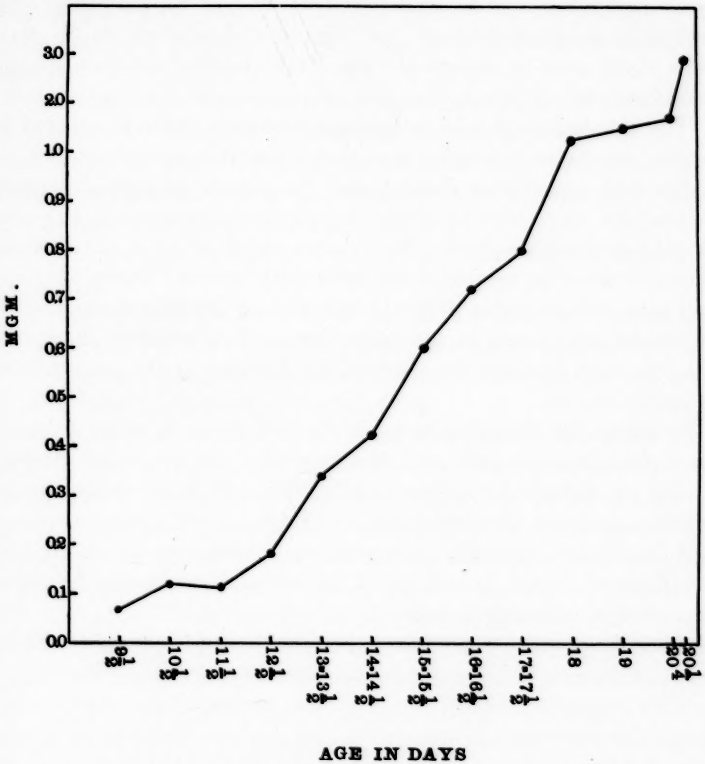

FIG. 6.—Total epinephrin content per pair of adrenal glands

Rabl (1891) and Hays (1914) studied the morphogenesis of the adrenal glands in the bird, but the physiological significance has remained untouched. By means of the cat's ilium experiment, Hogben and Crew (1923) reported that the epinephrin contents increase after 15 days of incubation. My data showed the epinephrin contents present in the gland from $9\frac{1}{2}$ days of incubation. In some cases, the value of epinephrin concentration per gram of gland tissue seems to

show a coincident dropping-off with the iodine content in the thyroid gland. Under this circumstance, the thyroid alteration might be supposed to have an effect on the metabolism in general, on the one hand, and is followed by change in the adrénals, on the other; or a reciprocal reaction between the thyroid and adrenal glands may very likely exist in embryonic life. This question requires further investigation.

The metabolism of calcium has long been believed to be controlled by the parathyroid. I found no evidence that this is true in embryonic life. My experiment showed that the parathyroid gland retains its cordlike structure throughout its whole embryonic life. It is obviously an inactive gland. The calcium metabolism in the embryo therefore must be controlled by some other means. Dieterle (1906) and some others found athyroid followed by imperfect ossification of the skeletal system in the human fetus. It is possible, therefore, that the thyroid takes the place of the function of the parathyroid in embryonic life.

Based on the foregoing data, the thyroid function seems to show more significance in embryonic life than other contemporary glands.

The physiological significance of the thyroid is attributed to an iodine-containing chemical body, the thyroxin. According to Kendall (1918), the thyroxin is essential as a catalyzer, regulating the fundamental chemical reaction of the body. If we accept the idea that growth is merely a response to a chemical stimulus, then the thyroxin is essential to stimulate the growth of an animal. Its function either above or below the normal level produces abnormality.

With regard to the thymus gland, a morphological observation shows the demarcation into cortex and medulla and Hassall's corpuscles after 15 days of incubation. The function of this gland, if any, must commence at the very latter part of the embryonic life.

SUMMARY AND CONCLUSIONS

Freshly laid Leghorn eggs were incubated. The morphology of the thyroid, parathyroid, adrenal, and thymus glands from the very beginning of incubation to the hatching period was observed. The iodine content of the thyroid glands and the epinephrin content of the adrenals at 24-hour intervals from $9\frac{1}{2}$ days of incubation to the

hatching period were determined. The significant results were as follows:

1. The vesicles in the thyroid glands were found from 9 days of incubation. Their active principle began to be detectable at the tenth day. Thereafter it increased in both absolute and relative amount.

2. The epinephrin content in the adrenal glands was detected from $9\frac{1}{2}$ days of incubation. It increased in amount definitely.

3. At 17–18 days of incubation both the iodine content of the thyroid and the epinephrin concentration of the adrenal gland tissue diminished. The reason was uncertain.

4. The parathyroid retained its cordlike structure throughout its embryonic life.

5. The thymus glands showed the demarcation into cortex and medulla and Hassall's corpuscles after 15 days of incubation.

6. The percentage of active principles of both glands reaches a normal level immediately after hatching.

These findings lead to the following conclusion: The active principle of the thyroid glands in the embryo increases in both the absolute and relative amount, and the latter indicates that the growth of the active principle is greater than that of the gland tissue. The epinephrin content of the adrenal glands increases in both the absolute and relative amount. The increase of relative amount determines the degree of increase in the cortical and medullary tissue per unit weight of gland. There seems to be an interrelation or reciprocal relation between the thyroid and adrenal glands. The percentage of active principles of the gland tissue of both the thyroid and adrenal glands reaches a normal level immediately after hatching.

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RESULTS OF FEEDING THYROID GLANDS OF VARIOUS TYPES OF VERTEBRATES TO TADPOLES[1]

(Three plates and three figures)

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INTRODUCTION

DURING the past several years considerable work has been done on the relationship of the thyroid gland to metamorphosis in the tadpole, and experiments have been performed in the feeding of both thyroid gland itself and pure iodine to both normal tadpoles and to specimens with the thyroids removed. The present paper records the results of experiments undertaken to determine the effect on tadpoles of the feeding of thyroids from different types of vertebrates and to determine whether or not this effect is correlated with the amount of iodine contained in the different thyroids. The experiments were started at the University of Kansas; but, owing to the removal of the writer, the later ones were carried on at the University of Washington (Seattle), where all of the measurements were made and the results tabulated.

LITERATURE

Little has been written that can be considered in the nature of a study of the comparative effects of feeding different kinds of thyroid glands to tadpoles, most of the literature on the relationship of the thyroid to the metamorphosis of tadpoles covering other phases of the subject. However, thyroids from several different kinds of animals, and glands from the same species but of different iodine content, have been fed, and some comparative work has been done.

Gudernatsch (1912 and 1914), the first person to record the fact that the feeding of thyroid hastened the metamorphosis of tadpoles,

[1] I wish to express my thanks to Dr. Bennet M. Allen, of the University of California at Los Angeles, under whose direction this work was carried on, for the help he has given me and for the many suggestions he has made as the work progressed and as this paper was in preparation.

fed horse thyroid to tadpoles of different ages, and also a mixed diet of thyroid and thymus. West (1914) fed bullfrog tadpoles Parke Davis and Company's[1] sheep thyroid tablets, with positive results; and during the same year, Morse, feeding *Rana pipiens*, used Parke Davis and Company's desiccated thyroid extract and Armour and Company's thyroid tablets, as well as fresh thyroid gland obtained at a packing house, but kind not specified—all with positive results. None of the foregoing experiments were carried on in a comparative way.

Lenhart (1915) fed dried and powdered human thyroids (both simple and exophthalmic goiters) and dog thyroid of various iodine content, but how he obtained these different iodine percentages is not stated. Also, he fed ox and sheep thyroid but did not record these results. In this case, the higher the percentage of iodine the more rapid was the metamorphosis of the tadpoles.

The following year, Graham fed tumorous human thyroid (adenomata) to the tadpoles of *Rana pipiens*, and found that the effect was closely related to the amount of iodine contained in the food, the same as in Lenhart's experiments. However, there were some exceptions to this, no explanation being given for them.

Gudernatsch (1917) later performed some experiments where thyroid and thymus glands were each split chemically into seven products and tadpoles were treated with the substances obtained. The thyroid nucleo-proteins caused the most rapid differentiation, and the precipitate from an alcoholic extract produced the greatest growth with the least differentiation. The other substances ranged between these two extremes in their action. Kendall (1917) isolated from the thyroid proteins an iodine-containing compound in pure crystalline form having a constant iodine content of 60 per cent, which he named "thyroxin." Rogoff (1918) reduced thyroxin still further and fed both the product of this reduction and the entire thyroxin to tadpoles. Both sets of specimens metamorphosed abnormally fast, those eating the thyroxin being slightly in the lead.

[1] Under date of December 10, 1919, Parke Davis and Company write: "Practically all of the supplies that we have been preparing for some time past are derived from the thyroid glands of hogs rather than those of sheep." A letter dated December 6, 1919, from Armour and Company says: "Our standardized powdered thyroid substance is made from the glands of sheep only."

Swingle (1918*a*) fed Armour and Company's powdered thyroid to tadpoles of *Rana pipiens*, with marked acceleration of metamorphosis resulting. The same author later (1919*a*) fed different quantities of iodine to tadpoles of *R. sylvatica* and found that the larger the amount the more rapid the transformation. Still later (1919*b*) he reported on the comparative effect of feeding iodine to normal tadpoles and to those with the thyroid gland removed. The results were the same in both instances, but it required a larger quantity to produce an equal effect in the case of the thyroidless tadpoles. The species used was *Bufo lentiginosus*. Finally, Swingle (1923) gave an extended discussion of the literature covering thyroid and iodine feeding, and, following experiments on the feeding of thyroidectomized and hypophysectomized frog larvae of *Rana sylvatica*, reaffirmed his belief that iodine is the substance essential for anuran metamorphosis.

Emmet and Allen (1919) fed tadpoles of *Rana pipiens* on diets of different kinds, the fat, carbohydrate, protein, and vitamine content of which were known. They were able to alter the size of the larvae and the rate of metamorphosis, but the latter process was not completed in any experiment. From experiments performed later (1920) by the same author and Sturtevant, the food used above was evidently practically free from iodine, because when this halogen, either organic or inorganic, was added to the diet, the tadpoles completed their metamorphosis.

The effect of feeding thyroid gland to tadpoles upon which thyroidectomy had been performed was reported by Allen (1917) as successful in one case in accelerating metamorphosis; and the same author (1919) caused similar results in both *Bufo* and *Rana* tadpoles from which both the pituitary and thyroid glands had been removed, by feeding them iodine mixed with flour.

Uhlenhuth (1921) gives a very good summary of the literature covering thyroid and iodine feeding experiments of the larvae of both frogs and salamanders.

Abderhalden and Schiffman (1922) fed human thyroid glands to tadpoles, and concluded that the physiological activity varied directly with the iodine content. However, in 1927, Abelin fed thyroid glands of calf and human embryos and of eighty-four cases of human goiter to tadpoles, checking on the iodine content in each

case. Four of the five calf thyroids used showed a fair amount of iodine content and caused metamorphosis. Iodine was found in the majority of the human embryo thyroids; but little, if any, influence upon the metamorphosis of tadpoles was produced by feeding this. As to goiters, those of the struma diffusa type had a high iodine content and strong biological activity; those of struma nodosa showed varied iodine values generally less than the preceding type, and showed less biological activity, but the latter was independent of iodine content; on the other hand, proliferating struma of Langhans and bone metastases of adenomas in the thyroid gland often showed a large amount of iodine and only a slight tendency to hasten metamorphosis in tadpoles. The conclusion was drawn that there is no necessary parallelism between iodine content and biological activity.

Cooksey and Rosenblatt (1928) fed human thyroid glands obtained from persons operated on for goiter to tadpoles of *Rana pipiens*, the iodine content of the glands fed being determined in each case. Thirteen separate feedings were reported on; and eleven showed results more or less closely correlated with the iodine content of the glands fed, while two did not. In these two exceptions, the biological activity was more than one would anticipate from the stated iodine content.

From the foregoing references it would appear that in practically all cases the feeding of thyroid to tadpoles will hasten their metamorphosis and that iodine will bring about the same result. Also, from the cases in which a comparative study was made, usually the biological activity of the thyroid gland is in direct proportion to the iodine content of the same. Graham (1916), when feeding human thyroid, recorded a few exceptions to this, as did Cooksey and Rosenblatt (1928); and Abelin (1927) alone draws the conclusion that there is no such relationship.

METHODS AND MATERIAL

The species used for the first series of experiments was the common bullfrog, *Rana catesbiana*. The specimens varied somewhat in size, but at the beginning of the experiments they were all less than a year old. The species used for the next series was the leopard frog,

R. pipiens. For the last series of experiments the tree frog, *Hyla regilla*, was used.

The experiments on the bullfrog were carried on in two rooms of different temperatures, other conditions being as nearly identical as was possible to obtain, in order to ascertain the effect of heat and cold on the rate of metamorphosis when accelerated by different kinds of thyroid feeding. Lenhart (1915) had reported that cold tended to delay the change.

A part of the series on *Rana pipiens* was also run on a comparative basis, but here the amount of water in which the separate experiments were kept was the variable quantity.

The remaining experiments were performed for the main purpose of the undertaking, namely, to ascertain the effect on tadpoles of the feeding of various kinds of thyroids and to see whether or not the results were correlated with the iodine content of the foods.

Regularity in the time of cleaning and feeding was maintained throughout the experiments. At 10 A.M. each day, the food not eaten during the preceding 24 hours was removed, and the water was changed. Then fresh food was placed on the surface of the water, and the tadpoles usually began to eat it at once.

Ten tadpoles were used as the unit in most of the experiments, being placed in a single container. In order to be sure of chance selections from the original stock of animals for each experiment each time a series was started, the tadpoles on hand were placed in four jars; and then one specimen was taken with a dip net from each jar in turn and placed in rotation in each of the containers making up the series. After this distribution the individuals were carefully measured and recorded.

The different series were started on the dates mentioned in each case and were closed from two to three days after the mortality had reached 30 per cent of the total number of specimens in the series in the case of *Rana catesbiana*, and 50 per cent in the other experiments.

The water used in the experiments performed in Kansas was Topeka city water, which comes from artesian wells. The water used at the University of Washington came from Lake Washington, which is fed by mountain streams. Both supplies are free from iodine.

The food used in these experiments was prepared as follows: Ordinary wheat flour, alfalfa flour, and powdered thyroid, which had been thoroughly dried and had had the fat removed, were mixed with water and rolled into thin sheets on glass. The wheat flour was run through a fine cheesecloth before using. In order to obtain alfalfa flour, alfalfa meal was run through either two or three cheesecloths of varying coarseness of mesh. The mixture was placed in the oven until thoroughly dry, peeled from the glass as flakes, and then fed.

Two different mixtures of these three flours were used, the one consisting of two parts wheat flour, two parts alfalfa flour, and one part thyroid, while the other was composed of two parts wheat flour, one part alfalfa flour, and one part thyroid. In the calculations due allowance for this difference has been made.

After being thoroughly dried, samples of the beef used as a control and of the various thyroids were analyzed in order to ascertain the iodine content of the same. The results follow:

	Percentage of Iodine
Beef (used in control)	0.0004
Calf thyroid (raw)	0.015
Raw beef thyroid (3 years old)	0.01
Cooked beef thyroid (mixed ages)	0.01
Pig thyroid	0.02
Dog thyroid	0.20
Commercial preparation A	0.40
Commercial preparation B	0.23
Human thyroid	0.009
Buffalo thyroid	0.053
Cat thyroid	0.008
Bear thyroid	0.025
Flounder thyroid	0.008

The preceding figures apply only to the material used in the following experiments, as there is a wide seasonal variation in the iodine content of the thyroid gland, as shown by the work of Seidell and Fenger (1914). All of the foregoing, except the commercial preparations, were collected during the summer months.

In the tables which show the results of the experiments on the tadpoles and the amount of food and of iodine consumed in each experiment, the following remarks are of uniform application. When meas-

uring the living tadpoles before the start of the experiments, three things were taken into consideration: the total length, the body length, and the length of the appendages. The forelegs were invariably absent; and the hind legs were also wanting in *Rana catesbiana* and in *Hyla regilla*, but were usually present in *Rana pipiens*, where they ranged from 0 to 1.05 mm. When it came to the examination of the specimens at the close of the different experiments, the foregoing items were again taken into account; and the individuals were dissected and the length of the stomach and of the intestine were recorded, and also the shape of the stomach and pancreas and the presence or absence of the pronephros and the urinary bladder.

In comparing the results of the different experiments with each other, it was found necessary to set up some more or less arbitrary rules by which they could be measured in order to determine the state or stage of metamorphosis reached by the specimens in the different units at the time they were closed, and from this to judge the rate of development. It was soon found that two standards would have to be taken—one for *Rana catesbiana* and another for *R. pipiens* and *Hyla regilla*. A discussion of the means used in comparing the different specimens will be found under the account of the experiments on each species.

In the tables covering the amount of food and iodine consumed, in order to obtain the amounts eaten per day, it was necessary to have some uniform time measure so as to calculate how long each experiment had run when compared to the others under discussion. This time-measure unit was obtained by adding the number of days each individual in a single experiment lived, and then dividing this number by the total number of individuals in the experiment, thus giving the average length of life, or the total number of days there was a full quota of specimens in each experiment.

With some individual exceptions, development of the forelegs is generally indicative of a change in the shape of the stomach and pancreas from that of the tadpole to that of the adult. Also, a similar statement can be made regarding the presence or absence of the pronephros and the urinary bladder, the former disappearing and the latter beginning to show a short time before the forelegs push out. In the tabulation of the results of the experiments these items

have been omitted, but it is assumed that the foregoing statements will be borne in mind when considering the results.

All the photographs were taken of specimens that had been preserved in formaline for some time. Where only one example from each experiment is shown, a typical or average-sized specimen was used. In the cases where the entire experiment was photographed, they were arranged in the order of their death, unless there was no variation in this, the ones dying first being placed on the left end of the row. All photographs are natural size.

EXPERIMENTS ON RANA CATESBIANA

Two experiments were performed on the tadpoles of *Rana catesbiana*, both being carried on in Topeka, Kansas, during the year 1920. As stated above, two objects were in view in running these experiments. The first of these was to find out whether or not the feeding of different kinds of thyroid would have identical effects on the rate of metamorphosis of the tadpoles, other conditions being equal. The second object was to ascertain what effect, if any, temperature had on the rate of metamorphosis of tadpoles when fed the different kinds of thyroid, other conditions being the same. The first experiment, here recorded, was carried on to determine the first objective; while the second one, reported later in this paper, was concerned primarily with the second objective.

Regarding the method devised for determining the relative state of metamorphosis reached at the time the different experiments were closed, undoubtedly all of the parts measured—that is, total length, length of body, of hind legs, of forelegs, of stomach, and of intestine—are of more or less importance; but it was early found that there was some fluctuation in total length and in the length of appendages due to individual variations. At the same time, it was seen that there was much less discrepancy between individuals from the same units as to stomach and intestinal lengths, but that out of these two, the more certain measurements could be taken on the intestine. If the specimens in a single experiment were classified according to length of intestine, placing the unit with the specimens having the shortest at one end of the list as those most nearly developed into frogs, and placing the experiment with the specimens

having the longest at the other end as indicating that they had passed through the least development, it was found that, with rare exceptions, the experiments and the individuals were listed in the same order as if each item of the measurements was ranked and counted. Of course, this applies only when comparing individuals or groups of the same species.

In these comparisons, however, one other factor besides the measurements of the different parts must be taken into account, that is, the date of death of the specimens, or, in other words, the length of time the experiment ran. It is evident that if all measurements were equal at the close of two parallel experiments, the specimens reaching this state of development in the shorter time, i.e., dying the sooner, would be considered as the more advanced. Taking this fact into consideration, it was decided that the best measure by which to judge the stage of development reached was to use the number resulting from the multiplication of the length of the intestine by the number of days the experiment ran as the criterion. This latter was determined by adding the number of days each individual in a unit lived, and then dividing the total thus obtained by the number of individuals in the unit; so the "days the experiment ran" is really the length of time covered if all specimens in the unit had died the day this average was reached, and not before or after as many did.

As a short intestine indicates an advanced stage of metamorphosis, and a small number of days during which the experiment lived usually denotes a rapid rate of development, it is evident that the smaller the number resulting from the multiplication of these two figures is, the more mature the specimen concerned must have been.

The first series was started with 130 bullfrogs (*Rana catesbiana*) on February 6, 1920. These were divided into sixteen groups of from six to ten specimens, and each group was placed in a single container. The room in which these experiments were carried on was heated until June 1, when the heat was turned off for the remainder of the summer. The measurements at the beginning and at the close of the experiments are shown in Table I, and the amounts of food and iodine eaten are recorded in Table II.

At the beginning of the experiments the specimens averaged very nearly the same in both total length and length of body, there being

no hind legs visible in any individual. At the close, they arranged themselves into two well-defined divisions. The first of these, with

TABLE I

SUMMARY OF MEASUREMENTS IN EXPERIMENTS ON *Rana catesbiana* BEFORE AND AFTER FEEDING
(Lengths in Millimeters)

Fed	Before Feeding			After Feeding						
	Total	Body	Hind Legs	Total	Body	Hind Legs	Forelegs		Stomach	Intestine
							Right	Left		
Control	56.8	23.6	.....	63.4	25.4	2.2	.....	.....	7.3	254.3
Raw beef thyroid	53.5	22.9	.....	63.2	24.8	1.5	.....	.....	8.8	260.6
Cooked beef thyroid	55.0	23.2	.....	64.1	24.7	2.4	.....	*	8.0	265.7
Pig thyroid	54.6	23.0	.....	59.5	24.2	0.5	.....	.....	7.8	240.1
Dog thyroid	55.8	23.5	.....	37.3	16.5	3.4	0.5	1.6	5.4	32.7
Commercial preparation A	55.0	23.6	.....	31.5	16.4	2.8	0.2	1.2	5.2	36.0
Commercial preparation B	53.5	22.9	.....	43.6	16.7	2.7	0.4	1.1	5.3	42.7

* Showing slightly on one specimen only.

TABLE II

AMOUNTS OF FOOD AND IODINE CONSUMED IN THE EXPERIMENTS ON *Rana catesbiana*
(Unit Used, Gram)

Fed	Amount Fed	Removed	Total Eaten		Days	Eaten Daily	
			Food	Iodine		Food	Iodine
Control	47.094	0.022	47.072	0.00004	195	0.2414	0.00000021
Raw beef thyroid	52.966	.047	52.919	.00100	204	.2594	.00000490
Cooked beef thyroid	51.263	.049	51.214	.00106	210	.2439	.00000505
Pig thyroid	40.025	0.065	39.960	.00167	204	.1959	.00000819
Dog thyroid	5.815	1.141	4.674	.00195	37	.1263	.00005270
Commercial preparation A	4.262	2.339	1.923	.00167	28	.0686	.00005928
Commercial preparation B	6.730	0.754	5.976	0.00285	38	0.1573	0.00007500

the index number representing the developmental stage reached in each case following, were those fed the control (4,959), raw beef thyroid (5,316), cooked beef thyroid (5,630), and pig thyroid (4,898); the second, the remaining three units fed dog thyroid (121), com-

mercial preparation A (98), and commercial preparation B (162). In the first, those fed cooked beef thyroid led, followed by those fed raw beef thyroid, control, and pig thyroid, all four being reasonably well spaced. The sequence of the second division was first that fed commercial preparation B, followed by dog thyroid, and last by commercial preparation A. The last specimens were the smallest and showed the most marked development of the appendages and shortening of the intestine, all three of which features point to the most advanced stage of metamorphosis.

Turning to Table II, which shows the amount of food and iodine consumed by the tadpoles in each unit, it is evident from the amount of uneaten food removed that probably the first four groups were underfed if anything; and in the last three it would seem as if an excessive amount of food was fed, and therefore had to be removed. In fact, the first four groups were fed approximately ten times as much as the remaining three; and at the same time they ate practically all they were fed, while the last three mentioned left a large proportion of their food untouched. A similar condition so far as these particular foods were concerned existed in all the experiments performed, not only on *Rana catesbiana*, but also on *R. pipiens* and *Hyla regilla*. At the outset of a series, all the tadpoles would eat readily; but after a short time those being fed food containing a relatively high percentage of iodine would seem to stop eating, growing sluggish very rapidly, and, as metamorphosis advanced, would lie inert in the bottom of the container. This always resulted in a decided difference in the total amount of food eaten by units developing with abnormal rapidity and by those passing through the period of metamorphosis at a rate more closely approximating normal. The total amount of food eaten was also closely associated with the number of days the experiment ran; so we would expect the first four groups to have consumed much more than the last three because the former were fed for from six to eight times as long as the latter.

Regarding the total amount of iodine eaten in each experiment, we find that this same grouping did not exist but that a new one arose. Those fed commercial preparation B were well in the lead, with 0.00285 grams. The remainder, with the exception of the control, formed a middle group with considerable variation within it-

self, while the amount of iodine eaten by the control was very small. This change in grouping when the total amount of iodine eaten is considered was due partially to the total amount of food consumed, but more to the fact that there was a wide difference between the iodine content of the different foods, the last three being especially well provided with it.

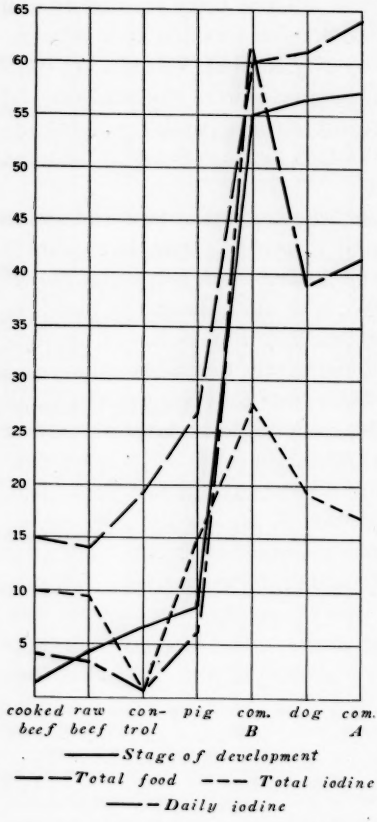


FIG. 1.—Experiments on *Rana catesbiana* showing comparative rates of development (determined in this species by multiplying length of intestine at the close by the number of days each experiment was carried on) and amounts of food and iodine consumed. The stage of development and the amount of iodine consumed increase with the figures on the left, while the reverse is true when the total food is considered.

When it comes to the question of the amount of iodine eaten daily, the last column indicates that the units fed dog thyroid and the two commercial preparations showed approximately ten times as much iodine eaten daily as any of the others. The amount eaten in the control was 0.00000021 gram, which was very much less than the others.

A comparison of Tables I and II, that is, the condition of the specimens at the end of the experiments with the amount of food and iodine consumed, is shown in Figure 1. In the graph, the different units are arranged so as to make a regular curve, with those showing the least development to the left. Both the total and daily iodine lines are drawn, so that the highest points indicate greater consumption. The total food line is the reverse. The

daily food record was omitted because it very closely coincides with the total amount consumed. It is evident that in this series of experiments there was a more or less close correlation among all these items. With slight variations, we have the least development associated with the largest total and daily amounts of food eaten and with the least amount of iodine consumed daily.

It appears that the total amount of iodine consumed had less to do with hastening metamorphosis, because the units fed commercial preparation A and dog thyroid consumed over half as much as that fed commercial preparation B, and yet both reached a stage of development which was slightly in advance of that reached by the latter. In comparing the rate of development with the amount of daily iodine consumed, it will be seen that there was a considerably closer correlation.

Photographs of typical specimens taken from each of the different feedings in these experiments are shown in Plate I. There was very little variation among the specimens in a single group.

EXPERIMENTS ON RANA PIPIENS

The experiments on *Rana pipiens* were carried on during the summer of 1920, the first being started June 20 and the last one closed July 28. Four separate experiments were run for the primary purpose of this paper, i.e., to determine the effect of feeding different kinds of thyroids to tadpoles of this species. Calf thyroid was added to the preparations previously fed. They were all conducted in the room in which the previously described experiments on *R. catesbiana* were performed, all the conditions being identical with the exception of the size of the containers. As the tadpoles of *R. pipiens* are much smaller than those of *R. catesbiana*, smaller dishes containing approximately 200 cc. of water were used. These experiments were repeated four times, and each unit consisted of ten specimens, making a total of 320 in all.

The tadpoles of *Rana pipiens* develop into frogs during the one season. Consequently a shorter time was necessary to complete a series of experiments. It will be noted that most of the specimens used had rudimentary hind legs present when the experiments started, a condition not true in *R. catesbiana*.

In determining the stage of development reached in the different experiments, it was found that the method used in the experiments on the bullfrog was not satisfactory in the case of *Rana pipiens*. At the start the tadpoles were small; and the first stage toward further development was an increase in size and consequently in the different parts, including the length of the intestine. Then after a certain period a peak in size was reached, and as further development was carried on, the measurements tended to decrease. Taking this into consideration, at the close of the experiments the different units were divided into two groups according to length of intestine, the first including the first five experiments, which were all evidently growing in size, and the second consisting of the last three, which had evidently passed the growing peak and were contracting. Of the measurements, the most constant and indicative seemed to be that of the hind legs. It, multiplied by the number of days, was taken as the criterion to show the comparative stage of metamorphosis reached. In the first group of five experiments, the smaller the number the less far advanced metamorphosis was; while in the second group, the reverse is probably true.

The results of the experiments on *Rana pipiens* are shown in Tables III and IV, and are graphically shown in Figure 2. Also typical specimens from each of the experiments are shown on Plate II.

From Table III, it would appear that the specimens were very uniform in the different units when the experiment started, and that the stages of development reached were also quite uniform in the two groups into which they were readily divided, as mentioned above. That is, assuming that the units with long intestines were still growing, and that those with short intestines were through with the growing stage and were approaching the completion of metamorphosis, by multiplying the length of the hind legs by the number of days the experiment ran, we obtain the index numbers given below that show the comparative stage of development reached in each unit. In the first group, the smaller number would indicate less development; while in the second group, the reverse would be true. Taking all this into consideration, they arranged themselves in the following order, beginning with the one showing the least development: that fed the control (47.4), followed by that fed raw beef thyroid (51.8), cooked

beef thyroid (60.1), pig thyroid (69.7), calf thyroid (72.2), commercial preparation B (19.2), dog thyroid (15.1), and commercial preparation A (12.7).

TABLE III

SUMMARY OF MEASUREMENTS IN THE EXPERIMENTS ON *Rana pipiens* BEFORE AND AFTER FEEDING
(Length in Millimeters)

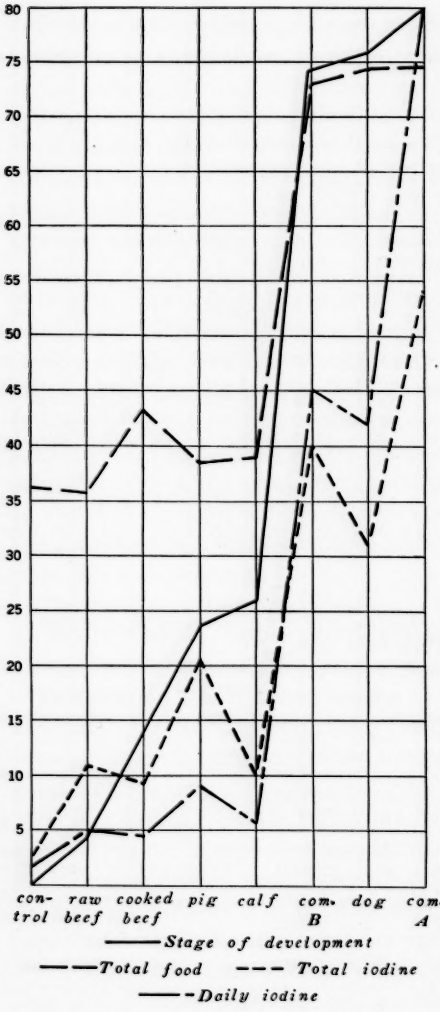
Fed	Before Feeding			After Feeding						
	Total	Body	Hind Legs	Total	Body	Hind Legs	Forelegs		Stomach	Intestine
							Right	Left		
Control	15.01	6.23	0.72	14.13	6.46	2.37	0.69	0.71	2.22	23.46
Calf thyroid	14.43	6.35	.61	15.12	7.47	3.44	1.18	1.16	2.38	29.57
Raw beef thyroid	14.17	6.09	.59	15.94	7.26	2.25	0.72	0.63	2.14	32.77
Cooked beef thyroid	14.08	6.18	.59	14.46	6.97	2.86	1.04	1.05	2.51	28.84
Pig thyroid	14.55	6.26	.61	14.18	6.73	3.17	0.82	0.93	2.15	25.92
Dog thyroid	14.68	6.26	.65	9.33	5.59	2.15	1.10	1.46	1.82	10.35
Commercial preparation A	14.48	6.31	.64	7.88	4.73	1.82	0.15	1.00	1.57	7.37
Commercial preparation B	14.52	6.16	0.60	8.55	4.77	2.13	0.69	1.05	1.57	6.92

TABLE IV

AMOUNTS OF FOOD AND IODINE CONSUMED IN EXPERIMENTS ON *Rana pipiens*
(Unit Used, Gram)

Fed	Amount Fed	Removed	Total Eaten		Days	Eaten Daily	
			Food	Iodine		Food	Iodine
Control	0.510	0.079	0.431	0.000001—	20	0.02007	0.000000020
Calf thyroid	.551	.031	.420	.000012	21	.02000	.000000571
Raw beef thyroid	.464	.028	.436	.000011	23	.01869	.000000473
Cooked beef thyroid	.413	.049	.364	.000009	21	.01597	.000000394
Pig thyroid	.447	.019	.428	.000021	22	.01838	.000000914
Dog thyroid	.097	.036	.061	.000031	7	.00840	.000004214
Commercial preparation A	.102	.048	.054	.000054	7	.00788	.000007880
Commercial preparation B	0.118	0.050	0.068	0.000040	9	0.00773	0.000004495

Turning to Table IV, it will be seen that the total amount of food eaten is fairly uniform in the first five experiments, but much less in the last three, this being roughly related to the number of days the specimens lived and the experiments were carried on. As to the total



amount of iodine consumed, those fed dog thyroid and commercial preparations A and B led by a considerable margin; while those fed on raw and cooked beef thyroid, calf thyroid, and the control ate a much smaller amount. As to the total amount of food eaten, as in the case of the *Rana catesbiana* experiments, the last three consumed much less than the first five. On the other hand, the daily iodine consumption by these last three was conspicuously heavy. Those fed calf thyroid, raw and cooked beef thyroid, and pig thyroid were much less, while the control consumed an insignificant amount per day.

In regard to the relationship of the rate of development to the material eaten, we consult both Tables III and IV and also Figure 2. In the latter, the average rate of de-

FIG. 2.—Experiments on *Rana pipiens* showing comparative rates of development (determined in this species by multiplying the length of hind legs at the close by the number of days each experiment was carried on) and amounts of food and iodine consumed. The stage of development and the amount of iodine consumed increase with the figures on the left, while the reverse is true when the total food is considered.

velopment for all the experiments on *Rana pipiens* is shown; they are arranged so that the line representing this will form a regular curve. The curve for the daily food consumption was omitted because it follows so closely the total food line that the one may be considered as standing for both items for all practical purposes. The lower numerals on the graph stand for slower rate of development and a less amount of iodine consumed. Regarding the food, the reverse is true, the lower number standing for the larger amount eaten.

There appears to be very little relationship between the amount of food eaten and the rate of development. There is less uniformity between these two items in the series under discussion than was found in the experiments on *Rana catesbiana*.

There is a very close correlation between the rate of development and the amount of iodine, both total and daily. That is, the more iodine consumed, the more rapid the rate of metamorphosis. In the total amount of iodine consumed, there is one rather marked break in the regularity of the line in the case of the experiment fed pig thyroid. On the other hand, the lines of comparative development and that of daily iodine consumption run remarkably alike, very slight exceptions to the general rule occurring in the cases of those fed on calf and dog thyroid. But these exceptions are so slight that they do not affect the conclusion that there is a close correlation between daily iodine consumption and rate of metamorphosis in the case of the tadpoles of *Rana pipiens*. This correlation is even clearer here than in the case of the experiments on *R. catesbiana*.

EXPERIMENTS ON HYLA REGILLA

The experiments on *Hyla regilla*, the tree frog, were carried on during the summer of 1922 at the University of Washington, Seattle, Washington. Their purpose was to ascertain further the effects of feeding the different kinds of thyroid previously used, to add to these material from other vertebrates, and to study the reactions of a new genus to thyroid feeding. The conditions under which the experiments were performed were as nearly identical with those for the ones that were done in Kansas as it was possible to make them. In all, four experiments were performed on this species; and units of ten tadpoles were used in each case except the last one, where twice

this number were fed. Several new kinds of food were used in these experiments, and all the varieties used previously were tried at least once. The new kinds were human thyroid, buffalo thyroid, cat thyroid, bear thyroid, and flounder thyroid.

The same method of determining the stage of development reached at the end of the experiments was followed as in the previous series on *Rana pipiens*, that is, the average length of hind legs attained was multiplied by the number of days the experiment ran, and the number resulting was taken as the index figure for stage of development reached. As in *R. pipiens*, a peak of development (determined by length of intestine) was reached; and until this took place, an increase in the index figure indicated greater development; but after the peak was passed, the opposite was true, i.e., the larger the number, the less the development attained. Hence, in the list given below, for the first six units the smaller number represents the lesser development, while for the last seven units the opposite is true.

TABLE V

SUMMARY OF MEASUREMENTS IN THE EXPERIMENTS ON *Hyla regilla* BEFORE AND AFTER FEEDING
(Length in Millimeters)

FED	BEFORE FEEDING			AFTER FEEDING						
	Total	Body	Hind Legs	Total	Body	Hind Legs	Forelegs		Stomach	Intestine
							Right	Left		
Control	17.01	7.21	.....	18.81	7.82	0.17	.....	.....	2.73	29.65
Calf thyroid	16.23	6.79	.....	19.81	7.71	.35	.....	.....	2.35	44.50
Raw beef thyroid	16.71	7.01	.....	17.88	7.62	.50	.....	.....	2.39	41.08
Cooked beef thyroid	16.97	6.92	.....	17.20	6.88	.29	.....	.....	2.16	36.05
Pig thyroid	16.54	7.09	.....	19.55	7.73	.43	.....	.....	2.42	44.40
Dog thyroid	16.60	7.17	.....	5.88	3.21	.55	0.16	0.15	1.18	4.70
Commercial preparation A	16.88	6.90	.....	8.22	4.14	.55	.....	0.17	1.41	7.11
Commercial preparation B	16.39	7.14	.....	8.95	4.29	0.62	.....	.....	1.31	6.30
Human thyroid	16.36	7.12	.....	11.29	5.22	1.03	0.05	0.07	1.72	10.84
Buffalo thyroid	17.09	7.37	.....	10.62	5.16	0.99	.....	0.06	1.69	9.14
Cat thyroid	16.62	7.14	.....	15.37	5.60	1.47	.....	.....	1.94	13.21
Bear thyroid	16.38	7.10	.....	10.08	5.08	1.09	0.12	0.21	1.60	6.56
Flounder thyroid	16.20	6.87	.....	17.49	6.84	0.42	.....	.....	1.57	31.74

The results of the experiments on *Hyla regilla* are shown in

Tables V and VI, and are graphically shown in Figure 3. Also, typical specimens are shown on Plate III.

From Table V, it will be seen that there was a wide range of development among the specimens in the different units at the close of the experiments. Taking into consideration what was said above regarding the attainment and passing of a development peak, the following is a list of the experiments in order from the least to the most developed at the close of the experiments, together with their comparative index figures in each case: control (4.42), followed by those fed cooked beef thyroid (6.67), calf thyroid (8.05), raw beef thyroid (8.50), flounder thyroid (8.82), pig thyroid (9.89), cat thyroid (35.28), bear thyroid (22.89), buffalo thyroid (19.80), human thyroid (18.54), commercial preparation B (8.06), dog thyroid (6.60), and commercial preparation A (5.50). There was a remarkably slight difference in the rate of development in the corresponding units of

TABLE VI

AMOUNTS OF FOOD AND IODINE CONSUMED IN EXPERIMENTS ON *Hyla regilla*
(Unit Used, Gram)

FED	AMOUNT FED	REMOVED	TOTAL EATEN		DAYS	EATEN DAILY	
			Food	Iodine		Food	Iodine
Control.....	0.420	0.110	0.310	0.000001	26	0.02059	0.00000017
Calf thyroid..	.311	.075	.236	.000003	23	.01026	.00000130
Raw beef thyroid.....	.385	.102	.283	.000006	17	.01608	.00000317
Cooked beef thyroid....	.296	.105	.191	.000004	23	.00830	.00000174
Pig thyroid...	.196	.081	.115	.000005	23	.00500	.00000274
Dog thyroid..	.164	.082	.082	.000033	12	.00683	.00002750
Commercial preparation A.....	.220	.155	.065	.000052	10	.00654	.00004828
Commercial preparation B.....	.208	.167	.041	.000014	13	.00315	.00001445
Human thyroid.....	.221	.074	.147	.000003	18	.00758	.00000136
Buffalo thyroid.....	.235	.128	.107	.000011	20	.00567	.00000601
Cat thyroid..	.209	.041	.168	.000003	24	.00727	.00000116
Bear thyroid.	.299	.135	.164	.000008	21	.00799	.00000399
Flounder thyroid.....	0.346	0.093	0.253	0.000004	21	0.01266	0.00000203

the different experiments, the least uniformity being shown by those fed human thyroid and bear thyroid.

Turning to Table VI, it will be seen that again, as in the previous

FIG. 3.—Experiments on *Hyla regilla* showing comparative rates of development (determined in this species by multiplying length of hind legs at close by number of days each experiment was carried on) and amounts of food and iodine consumed. The stage of development and the amount of iodine consumed increase with the figures on the left, while the reverse is true when the total food is considered.

experiments, as a general rule those having the slowest developmental rate consumed the most food, owing to the fact that they lived the longest. In both the cooked-beef-thyroid and pig-thyroid experiments, a slightly smaller amount than could be expected was eaten; while in the raw-beef-thyroid and human-thyroid experiments, the amount was slightly larger.

In Figure 3, as in previous experiments, the total and daily iodine consumption can be considered as one because there was very little difference between them, the two being almost parallel. In a general way, in this experiment, large iodine consumption went with fast development. But the units fed human thyroid and cat thyroid showed an exceptionally low iodine consumption associated with a more rapid rate of metamorphosis. It is possible that the active agent of the thyroid gland varies in intensity in different animals, and that the percentage of iodine contained in the gland is in a general way a gauge by which its action may be estimated, but that there are more or less marked exceptions to this and that probably the exceptions may extend in either direction from what might be called the "normal."

TEMPERATURE COMPARISONS

As stated above, eight units of *Rana catesbiana*, comprising eighty specimens in all, were run parallel to the foregoing experiments on the same species under identically similar conditions except that from February 6, the date of starting, to June 1, the former were conducted in an unheated room while the latter were carried on in a room supplied with heat, and hence warmer and drier. By the end of May, both rooms registered practically the same, and from June 1 until the close of the experiments both were placed in the same room under identical temperature conditions. Tables VII and VIII give the extremes and averages of temperature for the two rooms. The difference between the temperature of the two rooms averaged 8.8° C. in February, 6.3° in March, 6.3° in April, and 3.2° in May, making an average of 5.2° difference for the whole period the two experiments were separate.

The object in carrying on these additional experiments was to ascertain whether or not temperature had any effect on the rate of metamorphosis of frogs fed the different kinds of thyroid. Lenhart (1915) had previously reported that cold tended to delay metamorphosis in frogs being fed human and dog thyroid. At the same time, they were used as an additional check on the first set of experiments. The equipment used and the material fed were identical with those of the other experiments on this species.

In studying Table IX, which shows the measurements in these cold units both before and after the experiments were performed, it will be seen that almost everything that was said concerning the results of the previous experiments as shown in Table I can be repeated here with slight variation. The specimens were very uniform in size at the opening of the experiments, with no hind legs visible in any case.

TABLE VII

EXTREMES AND AVERAGE OF TEMPERATURES IN UNHEATED ROOM

Month	Low	High	Average
February	5.5	19.0	13.1
March	3.0	22.5	14.2
April	5.0	22.0	15.2
May	16.0	27.0	20.9

TABLE VIII

EXTREMES AND AVERAGE OF TEMPERATURES IN HEATED ROOM

Month	Low	High	Average
February	17.0	27.5	21.9
March	15.0	26.0	22.5
April	14.0	28.0	21.5
May	20.0	27.5	24.1

As occurred in the former experiments, the units grouped themselves into two divisions, the first five being in the one where the least metamorphosis occurred and the last three being in a group where metamorphosis was well advanced. Again taking the product of the length of intestine times the number of days the experiments ran as the criterion for judging the state of development, it will be seen that the order, beginning with the experiments showing the least development was as follows: those fed cooked beef thyroid (5,092), followed by the control (4,239), raw beef thyroid (3,918), pig thyroid (3,503), dog thyroid (291), commercial preparation B (278), and commercial preparation A (261). Comparing this order to that of the former experiments on *Rana catesbiana* recorded above, we find the two very

similar except that adjoining experiments are reversed in two cases, as before those fed raw beef thyroid preceded the control, and commercial preparation B was before the dog thyroid. These differences have little significance, however, as the stages of development reached in each of these units was not markedly different from the other one involved.

The comparison of the two groups of specimens fed the control, raw beef thyroid, cooked beef thyroid, and pig thyroid under the

TABLE IX

SUMMARY OF MEASUREMENTS IN EXPERIMENTS ON *Rana catesbiana* PERFORMED IN COOL ROOM BEFORE AND AFTER FEEDING

(Length in Millimeters)

FED	BEFORE FEEDING			AFTER FEEDING						
	Total	Body	Hind Legs	Total	Body	Hind Legs	Forelegs		Stomach	Intestine
							Right	Left		
Control	52.5	22.9	.....	61.6	24.9	1.3	.....	.....	8.3	236.6
Raw beef thyroid	53.7	22.6	.....	60.4	24.1	1.2	.....	.....	8.1	227.8
Cooked beef thyroid	54.3	23.2	.....	62.2	24.6	1.2	.....	.....	9.6	282.9
Pig thyroid	52.6	22.9	.....	55.6	22.6	0.2	.....	.....	7.2	212.3
Dog thyroid	53.0	22.9	.....	40.3	17.7	5.1	1.6	2.6	5.6	35.5
Commercial preparation A	54.4	23.4	.....	35.1	16.7	2.6	0.7	1.3	4.5	37.8
Commercial preparation B	52.6	22.4	.....	37.0	16.8	4.6	2.2	2.5	4.6	30.9

two temperature conditions show practically no difference in state of development reached at the end of the experiment. This was undoubtedly due to the very slow rate of development in both cases. Also, comparing the last three feedings in Tables I and IX, it will be seen that the specimens in each instance averaged very nearly the same in total size and size of various parts both at the beginning of the work and at its close. But it will be noted by Tables II and X that the units fed dog thyroid and commercial preparations A and B lived approximately three times as long as did their companion experiments in the preceding units. In other words, it took them three times as long to reach the same stage of development. From these same tables, we find that these last three units ate approximately twice as much total food and total iodine as in the corresponding

units of the first experiment; but, owing to the longer time they ran, their daily consumption was only about two-thirds that of the former.

From these facts the conclusion can be drawn that a lower temperature delays development of tadpoles, but when different kinds of thyroid material are fed, it does not perceptibly change the comparative rate of development. In short, a lower temperature delayed all the experiments at approximately the same rate regardless of what they were fed.

TABLE X

AMOUNTS OF FOOD AND IODINE CONSUMED IN EXPERIMENTS ON *Rana catesbiana* PERFORMED IN COOL ROOM

(Unit Used, Gram)

FED	AMOUNT FED	REMOVED	TOTAL EATEN		DAYS	EATEN DAILY	
			Food	Iodine		Food	Iodine
Control.....	37.680	0.025	37.655	0.00003	180	0.2092	0.00000017
Raw beef thyroid.....	35.965	1.619	34.344	.00069	172	.1997	.00000401
Cooked beef thyroid.....	37.495	1.002	36.493	.00073	180	.2027	.00000406
Pig thyroid....	24.352	1.636	22.716	.00091	165	.1376	.00000552
Dog thyroid...	7.852	0.888	6.964	.00279	82	.0849	.00003402
Commercial preparation A.....	7.178	4.590	2.588	.00207	69	.0375	.00003000
Commercial preparation B.....	10.344	1.691	8.653	0.00398	90	0.0961	0.00004422

EFFECT OF VARYING AMOUNTS OF WATER

Eight units comprising eighty specimens of *Rana pipiens* were run as parallel sets of experiments in order to ascertain whether or not the volume of water used had any effect on the results. As it had been discovered that the effects of feeding dog thyroid and the two commercial preparations were the most rapid in causing premature metamorphosis, these three were chosen as the foods for this comparative study. The conditions under which these were carried on were identical with the preceding series on *R. pipiens* except for the volume of water used. One set of these experiments was kept in shallow dishes containing 200 cc. of water, and the other set was in dishes containing 1,000 cc. The latter containers were larger than

the former and so the depth of water was practically the same in both. The water was carefully measured each day as changed. They were started July 3, 1920, and finally closed July 14. However, each corresponding pair except the controls were closed on the day when the last specimen in one of them died. That is, as soon as the last specimen in a unit died, the remaining specimens in the corresponding unit in the other series were killed. The dates of closing follow:

Controls on July 14, ten in 200 cc. and nine in 1,000 cc. killed.
Dog thyroid on July 10, ten in 1,000 cc. killed.
Commercial preparation A on July 11, ten in 1,000 cc. killed.
Commercial preparation B on July 14, nine in 1,000 cc. killed.

The results of these experiments are shown in Tables XI, XII, XIII, and XIV.

TABLE XI

SUMMARY OF MEASUREMENTS IN EXPERIMENTS ON *Rana pipiens* KEPT IN 200 CC. OF WATER BEFORE AND AFTER FEEDING

(Length in Millimeters)

Fed	Before Feeding			After Feeding						
	Total	Body	Hind Legs	Total	Body	Hind Legs	Forelegs		Stomach	Intestine
							Right	Left		
Control	14.41	6.26	0.39	15.83	7.15	0.49	.....	.....	2.80	45.78
Dog thyroid	14.55	6.23	.35	7.08	4.56	1.75	0.93	1.24	1.81	7.76
Commercial preparation A	14.07	6.10	.30	7.02	4.34	1.57	.31	0.95	1.65	5.26
Commercial preparation B	14.59	6.26	0.39	8.88	4.62	1.75	0.15	0.91	1.79	5.97

In comparing Tables XI and XIII, it will be seen that the specimens in the experiments were practically the same size at the start, and that the controls were in the same condition at the end. On the other hand, with the possible exception of length of intestine, all others except control in 200 cc. of water were markedly more mature in all respects than the corresponding ones in 1,000 cc. Also, the fact that the specimens in the experiments in 200 cc. of water, with the exception of the control, reached their maximum stage of possible development under the experimental conditions and died before the corresponding ones in 1,000 cc. did, would indicate the same thing.

TABLE XII

AMOUNTS OF FOOD AND IODINE CONSUMED IN EXPERIMENTS ON *Rana pipiens* KEPT IN 200 CC. OF WATER

(Unit Used, Gram)

FED	AMOUNT FED	REMOVED	TOTAL EATEN		DAYS	EATEN DAILY	
			Food	Iodine		Food	Iodine
Control	1.322	0.006	1.316	0.000001	11	0.11964	0.000000120
Dog thyroid	0.208	.064	0.144	.000072	7	.02057	.000010286
Commercial preparation A	.286	.109	.177	.000177	7	.02529	.000025286
Commercial preparation B	0.201	0.121	0.080	0.000046	10	0.00800	0.000004600

TABLE XIII

SUMMARY OF MEASUREMENTS IN EXPERIMENTS ON *Rana pipiens* KEPT IN 1,000 CC. OF WATER BEFORE AND AFTER FEEDING

(Length in Millimeters)

FED	BEFORE FEEDING			AFTER FEEDING						
	Total	Body	Hind Legs	Total	Body	Hind Legs	Forelegs		Stomach	Intestine
							Right	Left		
Control	14.58	6.36	0.37	13.26	6.58	0.52	.....	.....	2.42	38.30
Dog thyroid	14.25	6.10	.35	11.80	4.90	1.73	.....	0.10	1.83	6.23
Commercial preparation A	14.56	6.34	.49	10.37	5.05	1.66	0.02	.98	1.75	5.33
Commercial preparation B	14.43	6.31	0.37	10.95	4.84	1.65	0.29	0.52	1.75	5.33

TABLE XIV

AMOUNTS OF FOOD AND IODINE CONSUMED IN EXPERIMENTS ON *Rana pipiens* KEPT IN 1,000 CC. OF WATER

(Unit Used, Gram)

FED	AMOUNT FED	REMOVED	TOTAL EATEN		DAYS	EATEN DAILY	
			Food	Iodine		Food	Iodine
Control	0.354	0.123	0.231	0.000000+	10	0.02310	0.000000023
Dog thyroid	.185	.056	.129	.000064	7	.01843	.000009143
Commercial preparation A	.180	.092	.098	.000098	8	.01225	.000012250
Commercial preparation B	0.230	0.132	0.098	0.000056	11	0.00891	0.000005091

From these facts, it is evident that, with the exception of the control, those in 200 cc. of water developed more rapidly than did those in 1,000 cc. The figures on Plate III show the condition of the specimens at the end of the experiment.

Turning to the amount of food and iodine consumed in the corresponding experiments, as shown in Tables XII and XIV, it is seen that the 200 cc. series lead the 1,000 cc. in amount, with the exception of those fed commercial preparation B, where the reverse was true. In the case of those fed dog thyroid, however, this lead was so slight that it probably had little to do with the results; but the same cannot be said of the controls or of those fed commercial preparation A. In both these cases the units in 200 cc. consumed from two to four times as much as the corresponding ones in 1,000 cc., certainly enough to account for any more rapid development the former may have shown. It was hard to distinguish much difference between the specimens in the two controls at the close of the experiments. So it is evident that at least in the cases where dog thyroid and commercial preparation B were fed, it was not the greater amount of food consumed that caused the more rapid development in these experiments; it must have been the only other variable quantity, that is, the amount of water in which the specimens were kept.

Turning to a more detailed study of events in connection with these two series, after the first two days the experiments were carried on, with the exception of the controls, little food was consumed in any of the units. That is, almost all of the food actually eaten by the tadpoles was consumed during the first two days. After that, the specimens stayed on the bottom of the containers, motionless practically all the time except when disturbed. The flakes of food were placed on the surface of the water directly after it had been changed every morning at 10 o'clock. They floated there until they became waterlogged, when they sank to the bottom of the dish and remained until they were removed the following morning.

The question arises as to just why the specimens in the smaller amount of water developed so much faster than the ones in the larger amount. It seems to the writer that the only answer to this is to assume that some active substance which caused acceleration of metamorphosis in the tadpoles had dissolved out of the food, and

that this was naturally five times as concentrated in the experiments in 200 cc. of water as in those in 1,000 cc. It acted through the body wall and membranes of the tadpoles, its action being the same, with the possible exception of speed, as when taken into the alimentary canal. The specimens in the more concentrated solution would naturally react more rapidly than those in the weaker one. That the tadpoles of frogs and toads do not swallow water, but rather absorb it through the integument, is the contention of Boulenger (1914), which is in line with the foregoing conclusion. Also, Swingle (1919*b*) performed some experiments on the larvae of *Bufo lentiginosus* in which he did not vary the amount of water in which they were kept, but did place them in solutions consisting of different concentrations of iodine, with the result that those which obtained the most iodine metamorphosed the most rapidly. This would be in line with the present results, except that here it is not clear just what the active substance is that dissolved out of the food and caused the speeding-up of metamorphosis.

SUMMARY

In comparing the results of the different experiments, we find that the series fed the first eight kinds of food in each series, uniformly arranged themselves in two groups, the first showing a slower rate of development and the second a more rapid one. Invariably the units fed control, calf thyroid, raw beef thyroid, cooked beef thyroid, and pig thyroid were found in the first group; and those fed dog thyroid and the two commercial preparations were in the second. There was some variation in the arrangement of the series when different species were used, but it seemed to follow no rule. In the experiments on *Hyla regilla* where additional kinds of food were used, we find that the units fed flounder thyroid showed the slower rate of development characteristic of the first group above; while those fed human thyroid, buffalo thyroid, and bear thyroid had a distinctly rapid metamorphosis, thus placing them in the second group, although they all had a slower rate than those fed either dog thyroid or the two commercial preparations. The experiments fed cat thyroid were midway between these groups so far as rate of development went.

Regarding the relationship between the rate of development and

the amounts, both total and daily, of food eaten, it may be said that, with few exceptions, the greater amount of food consumed was associated with the slower rate of development. This is correlated with the length of time the experiments ran, as those living longer would naturally eat a greater amount, and the longer-lived experiments were the ones that developed the slowest.

As to the correlation between the amount of iodine consumed and the rate of development, it may be concluded that, with few exceptions, the greater the amount of iodine eaten the more rapid was the rate of development. Also, that if the percentage of iodine content is known, and equal amounts are fed, the food containing the most iodine will produce the most rapid metamorphosis regardless of its source. In the experiments on *Hyla regilla*, those fed human thyroid and cat thyroid showed a lower iodine consumption, together with a relatively rapid development. The reason for these exceptions to the general rule is not known; but they would indicate that possibly the active substance in the thyroid is not iodine itself, but rather some complex compound a part of which is iodine.

Parallel experiments on *Rana catesbiana* indicated that, while a low temperature slows up the process of metamorphosis, it has little or no effect in accelerating or retarding this process so far as the different foods are concerned.

Additional experiments on *Rana pipiens* showed that the amount of water in which the specimens were kept, like temperature, has an effect on the rate of metamorphosis, the less the amount of water the faster the rate; but that also, as in the case of temperature, there is nothing to indicate that it has a differential effect when the different thyroid foods are given. Apparently, the rate was accelerated in the units with less water because substances dissolved out of the food and were more concentrated when the water was in less quantity.

CONCLUSIONS

The following are the conclusions reached from a study of the foregoing experiments:

1. There is a close inverse correlation between the amount of food consumed and the rate of development in tadpoles—those eating the most develop the slowest. But this is what one would expect, be-

cause rapidly developing individuals live for a shorter time than those having a slower rate of metamorphosis; and as they eat for a shorter period, quite naturally will consume less food.

2. In experiments where thyroid is fed to tadpoles, the rate of metamorphosis is closely related to the amount of iodine consumed by the specimens—those obtaining the larger amounts, both total and daily, develop the more rapidly. Hence, if the percentage of iodine is known, the results of comparative feeding can be foretold.

3. The exceptions to the foregoing (2), seem to be associated with some specific kinds of thyroid. For example, human and cat seem to produce an abnormally rapid development when the small amount of iodine consumed is considered. This would indicate that the iodine alone is not the active agent of the thyroid gland, but that it is combined with, or is part of, the active agent and that this latter varies in composition in the glands of different animals.

4. A lower temperature tends to slow up the process of metamorphosis. However, there was little evidence that it affected the rate of development of the specimens fed one kind of thyroid any more than it did those fed other kinds.

5. The larger the volume of water in which the specimens were kept, the slower was the rate of development. This indicates that the active substance of the thyroid not only acts through the alimentary canal but also dissolves out of the food and affects the tadpoles through their integuments.

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PLATE I

Rana catesbiana

FIG. 4.—From experiment fed control (beef).
FIG. 5.—From experiment fed raw beef thyroid.
FIG. 6.—From experiment fed cooked beef thyroid.
FIG. 7.—From experiment fed pig thyroid.
FIG. 8.—From experiment fed dog thyroid.
FIG. 9.—From experiment fed commercial preparation A.
FIG. 10.—From experiment fed commercial preparation B.

The specimen shown in Figure 6 was dissected before the photograph was taken.

PLATE II

Rana pipiens

FIG. 11.—Specimens in experiment fed control (beef).
FIG. 12.—Specimens in experiment fed calf thyroid.
FIG. 13.—Specimens in experiment fed raw beef thyroid.
FIG. 14.—Specimens in experiment fed cooked beef thyroid.
FIG. 15.—Specimens in experiment fed pig thyroid.
FIG. 16.—Specimens in experiment fed dog thyroid.
FIG. 17.—Specimens in experiment fed commercial preparation A.
FIG. 18.—Specimens in experiment fed commercial preparation B.

In Figure 14, the first two specimens from the left were dried up.

PLATE III

Rana pipiens

The first four figures (19–22, inclusive) represent experiments that were carried on in containers holding 200 cc. of water, while the last four (23–26, inclusive) are photographs of similar experiments in which the amount of water was 1,000 cc.

FIG. 19.—Specimens in experiment fed control (beef).
FIG. 20.—Specimens in experiment fed dog thyroid.
FIG. 21.—Specimens in experiment fed commercial preparation A.
FIG. 22.—Specimens in experiment fed commercial preparation B.
FIG. 23.—Specimens in experiment fed control (beef).
FIG. 24.—Specimens in experiment fed dog thyroid.
FIG. 25.—Specimens in experiment fed commercial preparation A.
FIG. 26.—Specimens in experiment fed commercial preparation B.

In Figure 23, the first specimen from the left was dried up.

PLATE I

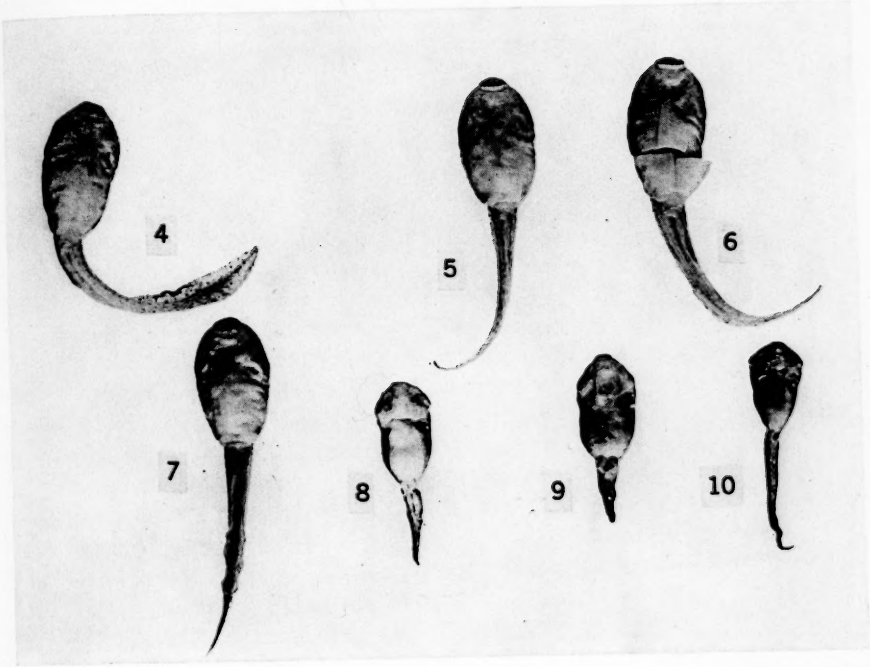


PLATE II

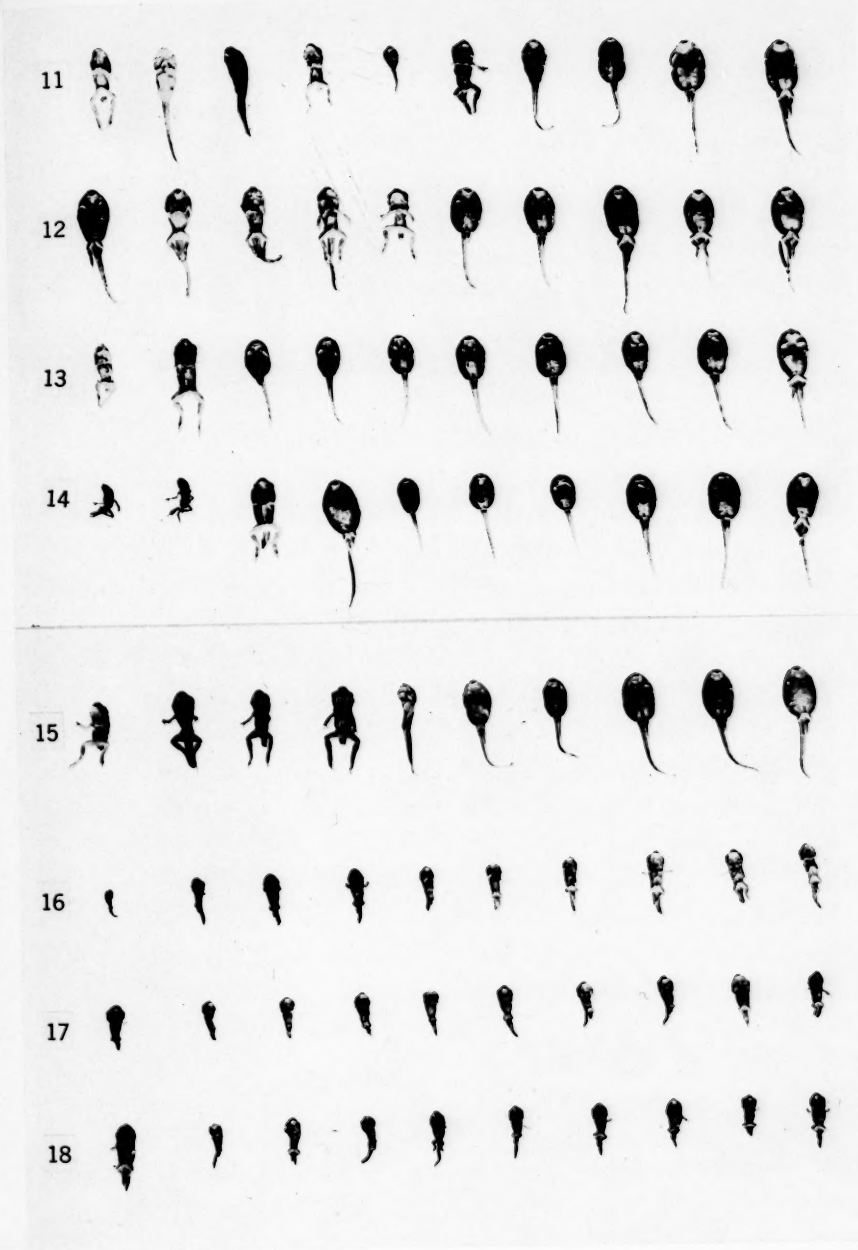
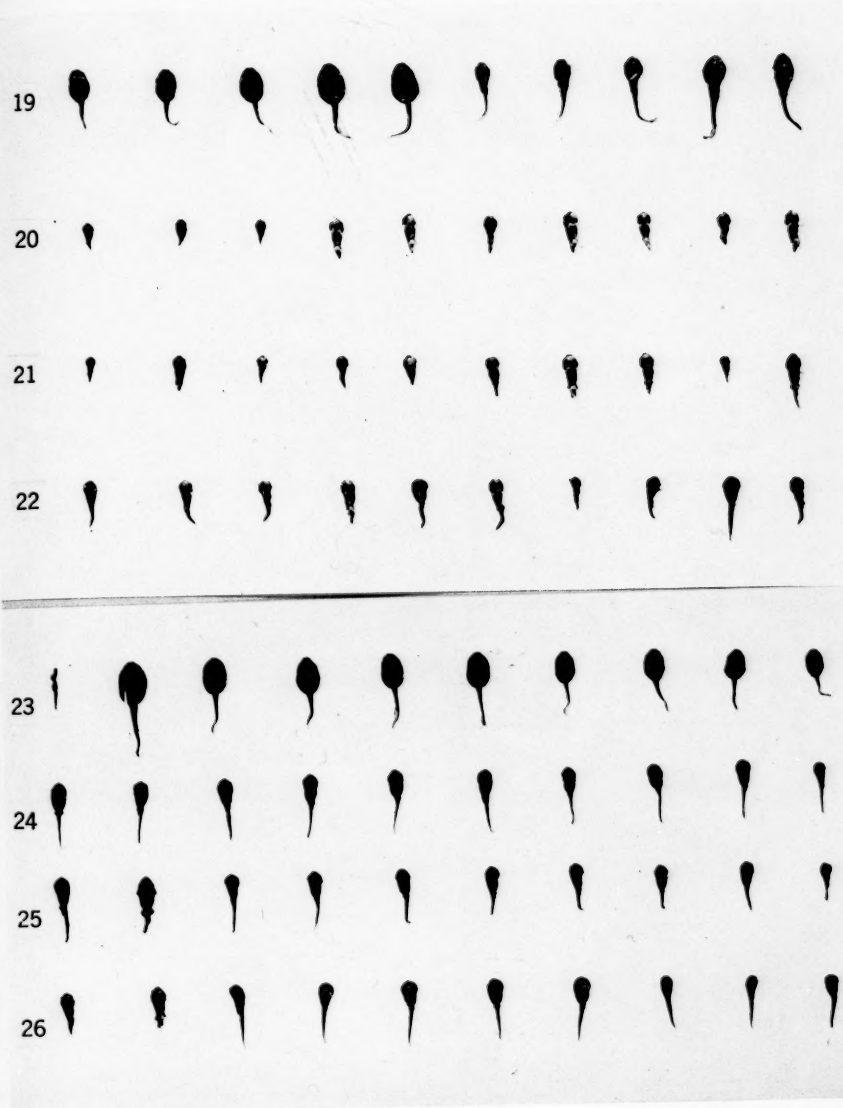


PLATE III



THE EFFECT OF TEMPERATURE ON THE GROWTH CURVES OF THE JAPANESE BEETLE (POPILLIA JAPONICA NEWMAN)

(Four figures)

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INTRODUCTION

THE present investigation deals with the changes in weight occurring in the life-cycle of the Japanese beetle at constant temperatures of 20° and 25° C. Ludwig (1928) found that the length of the larval instars is modified not only by temperature but also by food and the occurrence of a resting condition, the latter, in turn, depending on temperature. He showed that when larvae were fed only plant mold during the first instar, this so-called "resting stage" always occurred in the third instar at 25° C. At 22.5°, it occurred either in the second or third instar; at 20°, either in the first or second instar; and at 17.5°, always in the first instar. When the larvae were fed both wheat and plant mold from the time of hatching, the life-cycle was modified as follows: at 20°, the resting stage always occurred in the second instar; at 17.5°, either in the first or second instar. These studies demonstrated that the life-history at 20° is different from that at 25°. In order to determine the nature of the modification produced by this resting condition when it occurred in different stages of larval development, it was thought advisable to study the growth curves of the Japanese beetle at these temperatures.

MATERIAL AND METHODS

The eggs used in these experiments were collected in the laboratory from beetles brought in from the field during the latter part of August. Eggs were collected daily so that, when obtained, they were always less than 24 hours old. They were placed in sterile 1-ounce metal salve boxes in depressions made in moist, sterile soil. They were then divided into two groups, one being kept at 20° and the

other at 25° C. As soon as hatched, each larva was placed in a separate 1-ounce salve box containing plant mold. The salve boxes and plant mold had been previously sterilized in a hot-air sterilizer for 30 minutes at 150° C. In addition to the plant mold, each larva was given several grains of wheat to serve as food; and throughout the experiment an abundance of food was always available. Humidity was kept approximately constant (near the point of saturation) by the addition of tap water to the soil or plant mold. The larvae were kept at the same temperature as the eggs from which they hatched. In this way, records were obtained from individuals whose entire life-cycle—from egg to adult—was passed at 20° or 25° C. Temperature was kept constant to within 1° C.

Weight records were made, using a chainomatic balance sensitive to one-tenth of a milligram. To study the changes in weight during embryonic development, groups of 75 eggs were collected, weighed, and placed at 20° and 25° C. The eggs were weighed as a group, and the average was taken as the weight of a single egg. Daily weight readings were made throughout the period of embryonic development. The larvae were weighed individually, each within 24 hours of hatching, and then at intervals of 1 week, except just before a molt and during metamorphosis, when readings were made almost daily. In this manner, individual weight records were obtained from the time of hatching until the emergence of the adult.

To determine whether the increase in weight during embryonic development (Fig. 1) is due to imbibition of water, readings were made of the changes in water content of the egg at various stages of development. In this experiment 587 eggs were used. Water content was determined by weighing groups of 20–60 eggs, then dehydrating them in a hot-air oven at 100° C. for 4 hours and weighing again. The difference in weight is assumed to represent water lost by evaporation. From these figures the percentage of water contained in the eggs for each day of development at 20° C. was calculated.

OBSERVATIONS

Changes in weight and water content accompanying embryonic development.—The egg of the Japanese beetle, when laid, is elliptical and is relatively small, the average weight being only 0.86 milli-

grams. Within a few days it becomes spherical and increases to several times its original size. Figure 1 shows that there is a corresponding increase in weight to an average of 2.4 milligrams at the end of 8 days of incubation at 25°, and 14 or 15 days at 20° C. During the remainder of the embryonic period, the weight remains practically constant. Figure 1 shows that all phases of this growth period occur more rapidly at 25° than at 20° C.

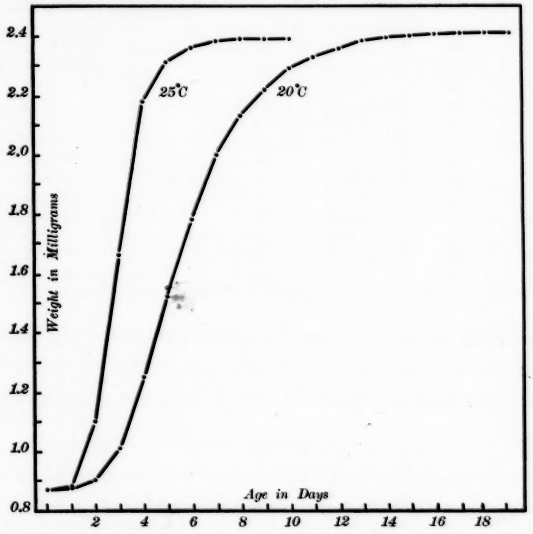

FIG. 1.—Changes in weight during embryonic development

The changes in water content which accompany the increase in size and weight of eggs kept at 20° are listed in Table I. The slight differences in weights given in this table and in Figure 1 are probably due to the fact that different groups of eggs were used in each experiment. Table I shows that the egg, when laid, contains an average of only 49 per cent of water. The water content increases to an average of 84 per cent at the end of 14 days of incubation at 20° C. The weight of water contained in the eggs at different stages of development was obtained by multiplying the percentage of water present in a given stage by the average weight obtained for that stage. Table

I also shows that the increase in water content is more than sufficient to account for the increase in weight. This allows for the slight loss of weight resulting from the metabolism of some of the egg substances.

TABLE I

INCREASE IN WEIGHT AND WATER CONTENT DURING EMBRYONIC DEVELOPMENT

Age at 20° C. (Days)	Number of Eggs	Average Weight (Milligrams)	Percentage of Water	Average Weight of Water (Milligrams)	Increase in Weight (Milligrams)	Increase in Weight of Water (Milligrams)
1	50	0.862	49.0	0.422	.....	.....
2	49	0.942	50.5	.475	0.08	0.053
3	46	1.039	55.9	.580	.177	.158
4	41	1.241	63.9	0.792	.379	.370
5	50	1.506	70.9	1.067	.644	.645
6	63	1.668	74.4	1.240	.806	.818
7	43	1.790	76.0	1.360	0.928	0.938
8	43	1.983	77.7	1.540	1.121	1.118
9	20	2.115	80.0	1.692	1.253	1.270
10	57	2.247	81.0	1.820	1.385	1.398
11	40	2.280	81.1	1.849	1.418	1.427
13	50	2.416	83.1	2.007	1.554	1.585
14	35	2.44	84.0	2.049	1.578	1.627

Growth curves at 25° C.—One hundred and ten individual records were obtained covering the entire growth period of the larvae. The results, summarized in Table II, fall into three groups, based upon

TABLE II

WEIGHT CHANGES DURING GROWTH AND METAMORPHOSIS AT 25° C.

(Weight Expressed in Milligrams)

Group	First Instar (Just Hatched)	Second Instar (Just Molted)	Third Instar (Just Molted)	Maximum Larval Weight	Early Prepupa	Early Pupa	Adult
1	2.4±0.02	17.1±0.16	89.9±0.83	282.1±2.68	263.9±3.03	241.4±3.5	143.3±3.87
2	2.2±0.05	13.0±0.29	68.1±1.4	221.8±4.57	209.2±5.93	199.8±8.68	119.7
3	2.5±0.06	13.4±0.57	51.5±1.58	164.5±5.19	144.9	144.4	.....
Average	2.35±0.019	16.5±0.17	86.1±0.93	270.8±2.90	254.5±3.08	235.6±3.61	150.4±3.52

the distribution of the growth curves about three means. There were 94 larvae in the first group, 12 in the second, and 4 in the third. The average daily increase in weight during growth, expressed in percentages, is shown in Figure 2. This figure shows that the high initial rate of growth is continued longer in Group 1 than in Groups 2 or 3; while in Groups 2 and 3, the growth period is longer than in

Group 1. Group 2 is intermediate between 1 and 3. In Group 1, the growth period is relatively short, the larvae becoming full grown in about 50 days. From then until the beginning of metamorphosis, weight remains approximately constant. In Group 2, the growth period averages approximately 65 days; in Group 3, approximately

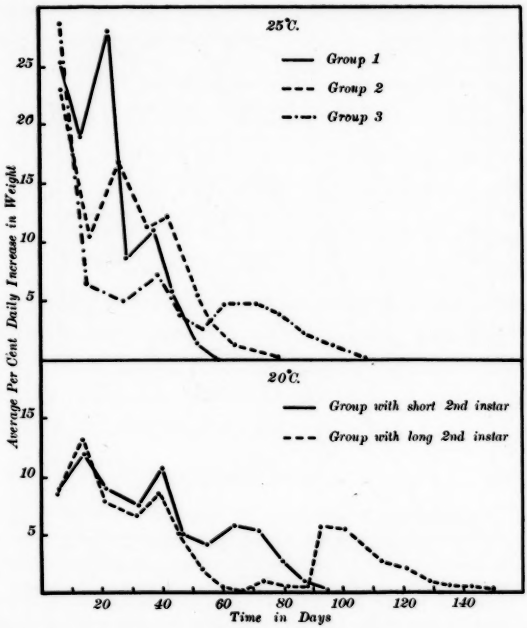

FIG. 2.—Average daily increase in weight of each group expressed in percentages

90 days. In these two groups, the resting period previous to metamorphosis is correspondingly shorter.

The changes in weight during growth and metamorphosis for an individual of each group are plotted in Figure 3. Each was selected because its growth curve corresponds very closely to the mean for that group. In order to eliminate sex differences, only males were selected. These curves show that, in the Japanese beetle, increase in weight occurs only between molts. A few days before molting, the larva stops feeding and most of the fecal material is eliminated. Dur-

ing this preparation for molting, there is usually a slight loss of weight. The fluctuations in the curves after the completion of growth are probably due to feeding and to defecation.

Figure 3 also shows that the first and second instars are shortest in Group 1 and longest in Group 3, while Group 2 is intermediate.

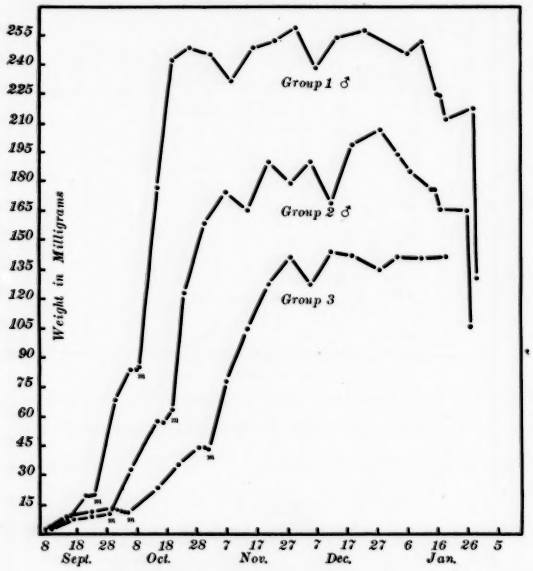

FIG. 3.—Changes in weight during growth and metamorphosis of an individual of each group at 25° C. Each was selected because its growth curve corresponds very closely to the mean for that group. *m* indicates the time of each larval molt.

These differences in the length of the various stages are summarized in Table III. The average figures for all three groups at 25° are practically identical with those obtained by Ludwig (1928, p. 368) under the same conditions of temperature and food. It is interesting to note that, while the length of the first and second instars is shorter in Group 1 than in Group 2, the total length of the larval period is practically the same, being 135 days in Group 1 and 132.5 days in Group 2. This also confirms the previous statement (Ludwig, 1928) that the length of the third instar is determined by the past history

of the individual, being relatively long when previous stages are relatively short. Not only does growth occur more rapidly in Group 1 than in the other groups but the individuals are heavier, as shown in Table II.

TABLE III

DURATION OF EACH LARVAL STAGE AT 25° C.

GROUP	FIRST INSTAR		SECOND INSTAR		THIRD INSTAR PLUS PREPUPA	
	Number of Larvae	Duration (Days)	Number of Larvae	Duration (Days)	Number of Larvae	Duration (Days)
1.....	94	15.6±0.14	94	16.9±0.17	48	102.5±1.1
2.....	12	21.5±0.83	12	19.6±0.41	5	91.4±3.34
3.....	4	27.5±2.19	4	35.2±2.39	1	106.
Average.	.....	16.7±0.24	.....	17.9±0.28	.....	101.5±1.09

Metamorphosis is accompanied by a regular loss of weight, beginning with the early prepupa and continuing until the emergence of the adult, except during the pupal stage, when the weight remains approximately constant. Table II shows that this weight loss is greatest during the change from pupa to adult.

TABLE IV

WEIGHT DIFFERENCES BETWEEN MALES AND FEMALES

(Weight Expressed in Milligrams)

	First Instar (Just Hatched)	Second Instar (Just Molted)	Third Instar (Just Molted)	Maximum Larval Weight	Early Prepupae	Early Pupa	Adult
Males.....	2.4±0.03	16.2±0.37	82.1±1.2	255.9±3.23	235.1±3.96	212.3±3.62	132.8±2.67
Females....	2.2±0.06	17.0±0.36	95.2±2.6	307.2±5.43	284.1±5.76	264.1±4.68	179.6±4.08
Average..	2.3±0.03	16.5±0.26	87.2±1.46	268.8±4.08	247.9±4.25	226.7±4.03	150.4±3.52

With data obtained from 38 adults (23 males and 15 females) reared at 25° C., an analysis was made of sex differences in weight appearing during growth and metamorphosis. These results are summarized in Table IV. No differences are apparent until the larvae have reached the second instar. However, these differences do not become statistically significant until the third instar. After the attainment of the maximum larval weight and throughout metamor-

phosis, the average weight of the females is approximately 50 milligrams greater than that of the males.

Growth curves at 20° C.—Eighty complete records were obtained covering the growth period of the larvae. At 20° they fell into two groups, depending on the length of the second instar. In one group of 32 individuals, the second instar averaged only 33.3 days; while in the other group, containing 48 individuals, it was relatively long, averaging 70.0 days. Some of the larvae appeared to be intermediate between those with long and those with short second instars. In this study, however, all the larvae in which growth had stopped tem-

TABLE V

DURATION OF EACH LARVAL STAGE AT 20° C.

GROUP	FIRST INSTAR		SECOND INSTAR		THIRD INSTAR PLUS PREPUPA	
	Number of Larvae	Duration (Days)	Number of Larvae	Duration (Days)	Number of Larvae	Duration (Days)
Short second instar.....	32	30.4±0.54	32	33.3±0.83	9	125.8±4.9
Long second instar.....	48	29.1±0.42	48	70.0±1.47	14	91.6±2.93
Average.	.....	29.6±0.33	.....	55.5±1.71	.....	105.0±3.5

porarily in the second instar were placed in the group with a relatively long second instar. Table V summarizes the time required for the completion of each larval instar at 20°. It will be noted that in the first group the third instar is relatively long, and in the second group it is relatively short. Here, as in the groups observed at 25°, the total larval stage is of approximately the same length, averaging 189.5 days in one group and 190.7 days in the other.

The weight changes during growth and metamorphosis at 20° are shown in Table VI. The average weight of the larva, immediately after hatching, is 2.3 milligrams. The average daily increase in weight, expressed in percentages, is plotted in Figure 2. An examination of this figure clearly shows the differences between the two groups. In the group with a short second instar, growth continues uninterrupted, except for molts, until the larvae are full grown. In the other group there is a period of about 5 weeks, represented by

the low part of the curve, in which growth practically ceases. After this period, growth occurs very rapidly until the larvae are full grown. This period of interrupted growth is the so-called "resting stage," which occurred in the second instar in those individuals with a long second instar. Growth curves for an individual of each group are plotted in Figure 4. Individuals were selected whose growth curves correspond very closely to the mean curve for each group. The difference in weights of the individuals whose growth curves are plotted is not to be interpreted as a weight difference between the two groups, but rather as a sex difference.

TABLE VI

CHANGES IN WEIGHT DURING GROWTH AND METAMORPHOSIS AT 20° C.

(Weight Expressed in Milligrams)

Group	First Instar (Just Hatched)	Second Instar (Just Molted)	Third Instar (Just Molted)	Maximum Larval Weight	Early Prepupa	Early Pupa	Adult (Just Emerged)
Short second instar....	2.3±0.035	13.2±0.31	61.7±1.77	227.6±5.99	211.4±7.56	200.0±10.6	111.2
Long second instar....	2.3±0.034	13.0±0.20	60.5±1.23	219.2±5.19	216.7±5.25	192.6±6.41	130.7
Average..	2.3±0.024	13.1±0.14	60.9±1.02	223.4±3.98	214.2±4.42	195.5±5.73	123.6±5.1

During metamorphosis, there also occurs a regular loss of weight which is essentially the same as that described at 25°, except that the weight of the pupa gradually decreases at 20° while at 25° it remains practically constant.

Eleven adults, 5 males and 6 females, were obtained. Although this number is too small to justify an analysis of the results on the basis of sex, the average weight of the female adults is approximately 40 milligrams more than that of the males. This is essentially in agreement with the results obtained at 25°.

DISCUSSION

A number of investigators—Przibram and Megusar (1912), Stzern (1914), Titschack (1924), Eidmann (1924*a* and *b*), and others—have stated that molting in insects occurs when the weight has doubled. This doubling of weight is supposed to result from a single division of the body cells. In case the weight is more than doubled, the ratio has

been found to be some exponent of 2, such as 2^2, 2^3, 2^4, etc. If the ratio of the weight increase from one molt to another is 4, it is supposed that two divisions of the cells have occurred; if it is 8, it is assumed that there have been three cell divisions. This explanation has been given by Przibram and Megusar (1912), Bodenheimer

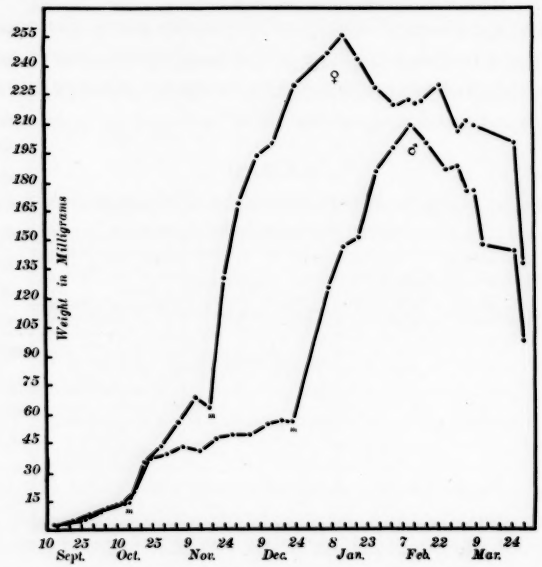

FIG. 4.—Changes in weight during growth and metamorphosis of an individual of each group at 20° C. Each was selected because its growth curve corresponds very closely to the mean for that group. *m* indicates the time of each larval molt.

(1927), and Alpatov (1929). The present investigation has failed to substantiate this idea. At 25° C. the ratio of the weight after the first molt to the weight of the newly hatched larva ranged from 4 to 12, with an average of 6.5; at 20° C. it ranged from 4 to 8, with an average of 5.7. The ratio of the weight immediately following the second molt to the weight immediately following the first molt, at 25°, ranged from 3 to 8, with an average of 5.2; at 20°, it ranged from 3 to 8, with an average of 4.6.

Calvert (1929) gives a very comprehensive discussion of growth in

insects with especial reference to increase in length. He speaks of "continuous and discontinuous growth," the latter being growth which occurs chiefly at intervals corresponding to the periods of molting. In these forms the growth curve resembles a series of steps. In regard to increase in weight, a similar subdivision may be made. Shafer (1922) found that in the dragon-fly nymphs, *Anax junius* and *Aeschna multicolor*, there is an abrupt increase in weight immediately following molts. He found that this weight increase amounts to 43.5 per cent of the weight of the nymph just before the molt and that the percentage increase in weight which occurs during the stage is always considerably less than the percentage increase which occurs at the beginning of the stage in question. Titschack (1924) also found that in the walking stick, *Carausius morosus*, the weight increase is greatest immediately following the molt. Similarly, Blunck (1924) stated that in *Dytiscus marginalis* the weight curve rises almost vertically following molting and then gradually becomes more and more horizontal and makes a new jump at the next molt. On the other hand, Figures 3 and 4 show that in the Japanese beetle increase in weight is continuous, except at the time of molting and in those individuals with a resting stage occurring before the completion of growth. Similar results have been obtained by Eidmann (1924*a*) and Teissier (1928).

The possibility of physiological varieties.—The results just described indicate that the Japanese beetle population of this country is composed of several physiological varieties which differ from each other in size and rate of growth under the same conditions of temperature and food. The separation into three groups at 25° and two groups at 20° is probably concerned with physiological characteristics which are quite different, since (1) the number of larvae in each group at 25° is different than at 20°, and since (2) at 25° there are significant differences in the weights of individuals in different groups (see Fig. 3 and Table II), while at 20° there are no such differences (see Table VI). It is probable that further experiments might justify a subdivision of each group at 20° on a basis similar to that used at 25°. There is also the possibility that the same physiological characteristics may be expressed in a different manner under different environmental conditions. During previous investigations on the Jap-

anese beetle, it has always been observed that, under the same environmental conditions, most individuals complete a particular larval stage at approximately the same time, that is, within a period of one or two weeks. However, there is always a small percentage of larvae which lag in development for several weeks, or even months, beyond the majority. The present work indicates that these individuals belong to a different physiological variety. An indication of similar physiological differences between individuals is found in the work of Alpatov (1929) on *Drosophila melanogaster*. He states, on page 420: "It must be mentioned that there are cases when single individuals remain far behind their sibs in their development. So, for instance, one individual 0.711 mm. long remained a larva of the second stage until 72.5 hours of age. But, in general, the change of the stage occurs at nearly the same time in all the larvae." This suggests that Alpatov may have been dealing with a condition similar to that described in the Japanese beetle. In the present experiments, the resting stage occurred in either the second or third instar at 20° C., while Ludwig (1928) found it to occur in either the first or second instar at that temperature. As far as could be determined, environmental conditions, such as food, humidity, and temperature, were identical in both experiments. The only known difference in the two series is the time of the year at which the eggs were collected. In the previous experiments, the larvae hatched from eggs which were laid in the early part of July; while in the present experiments, the eggs were collected during the last week of August. This suggests that season may also be a factor modifying the life-history of an insect. A comparable condition has been described by Heller (1926). He was able to distinguish two kinds of larvae in the lepidopteran, *Deilephila euphorbiae*, depending upon the length of time required for the development of the pupae which they produce. First, those producing pupae which at a temperature of 22°–23° C. show "subitane" development, that is, the pupal stage is completed in a relatively short time; second, those producing pupae which even at a temperature as high as 30° C. show "latente" development, that is, they must pass the winter in the pupal stage. The occurrence of these two types of larvae was found to be determined by season. In July

about 90 per cent of the larvae were of the first type, and as the season progressed the number became fewer.

To determine whether season is a modifying factor in the life-history of the Japanese beetle, eggs were collected at intervals from the first week of July until the last week of September. They were allowed to hatch at room temperature, and the larvae were then transferred to 20° C. Individual records of the duration of each larval instar were obtained.[1] The results on the second instar, summarized in Table VII, show that there is no correlation between the

TABLE VII

EFFECT OF SEASON ON THE DURATION OF THE SECOND INSTAR

Date When Beetles Were Collected	Date When Eggs Were Collected	Number of Larvae	Duration of Second Instar (Days)	Percentage of Larvae with Short Second Instar
July 5	July 8–11	55	60.1±2.28	36.3
July 5	August 6	39	67.9±2.36	17.9
July 19	July 23–26	42	65.3±2.72	30.9
August 16	August 19–20	52	63.6±1.88	33.3
September 7	September 11–15	22	68.6±2.21	9.0
September 19	September 22–28	8	55.1±4.84	37.5

time of egg-laying and the duration of this instar. In this experiment the resting stage always occurred either in the second or third instar.

Modification of growth by temperature.—It is a well-known fact that increase in temperature, within the limits of normal development, increases the rate of development and shortens the time required for the completion of the growth period. This is illustrated graphically in Figure 2, in which the average daily increases in weight at 20° and 25° C. are plotted. At 25°, values as high as 28 per cent were obtained for the first few weeks of larval development; while at 20°, the highest value obtained was 13 per cent. It is also interesting to note that when the resting stage occurred in the second instar, the curve shows two peaks, one at the beginning of larval growth and the other at the end of the resting stage. These results are comparable to those described by Calvert (1929) for the nymphs

[1] The writer is indebted to the College of Engineering of New York University for the use of a constant-temperature room during a part of this experiment.

of the dragon fly, *Anax junius*. In this species, growth ceases during the winter months, although the nymphs are kept at room temperature. The resumption of growth in the spring is also indicated by a second peak in the percentage growth curve.

An examination of tables II and VI shows that the maximum weight of the larvae is less at 20° than at 25° C. For example, the average maximum weight at 20° is 223.4 milligrams, as compared with 268.8 milligrams at 25°. Similarly, the weights of adults reared from larvae kept at these temperatures are 123.6 and 150.4 milligrams, respectively. Musconi (1924) found that at lower temperatures development of larvae of *Calliphora eritrocephala* took longer but that the larvae became larger than at higher temperatures. When reared at 8°–10° C., the larvae measured 19 millimeters; at 20°–24°, they measured 16 millimeters; and at 38°, 13–14 millimeters. Titschack (1925), in studying the influence of temperature on the development of the clothes moth, *Tineola biselliella*, found also that at high temperatures the larvae were smaller and lighter than at lower temperatures and that growth occurs more slowly at low than at high temperatures. Correspondingly, moths reared from larvae at high temperatures were smaller than those reared from larvae at low temperatures. From these experiments it is evident that temperature modifies the size and weight of insects and that the Japanese beetle is different from the species just cited.

Ludwig (1931) showed that, in respect to weight, the Japanese beetle is extremely variable, the male adults (within 24 hours after emergence) ranging from 82.3 to 152.9 milligrams and the females from 109.5 to 183.5 milligrams. The present study has shown that rearing the larvae at different temperatures increases this variability as follows: at 20°, minimum and maximum weights of male adults are 75.8 and 121.7 milligrams, of females 105.7 and 171.6 milligrams; at 25°, similar weights of males are 105.7 and 171.6 milligrams, of females 136.3 and 220.0 milligrams. Adult beetles thus range in weight from 75.8 to 220.0 milligrams. In the previous experiments (Ludwig, 1931) larvae were obtained in the field during March and placed at 25° C. until adults emerged. The weights of the adults thus obtained agree more closely with the weights of adults reared at 20° than those reared at 25°.

In the present experiments, the loss of weight occurring during metamorphosis was practically the same as that reported by Ludwig (1931), except during the pupal stage at 20°. At 25° and in the experiments just cited, which also were performed at 25°, there was a loss of weight of slightly more than 1 milligram during the entire pupal stage. This was explained as due to the absorption of water, the water content of the pupa increasing from 74 per cent in the early pupa to 76.8 per cent in the pupa just before emergence. However, at 20° there was a gradual loss of weight during the pupal stage which amounted to an average of 5.7 milligrams. A number of investigators, including Newport (1837), Blasius (1866), Herms (1907), and Bodine and Orr (1925), have also reported a gradual loss of weight during the pupal stage of various insects.

SUMMARY

1. On the basis of rate of growth and weight, the larvae of the Japanese beetle reared at 25° C. may be separated into three groups suggesting physiological varieties. At 20° C. they were separated into two groups: one with the resting stage in the third instar and, consequently, with a relatively short second instar; the other with the resting stage in the second instar which, therefore, was relatively long.

2. The occurrence of this resting stage in a particular larval instar is apparently not influenced by season. Larvae were hatched from eggs collected at intervals from the first week of July until the last week of September, and reared at 20° C. In these various groups there was no correlation between the time of egg-laying and the duration of the second instar.

3. When the resting stage occurs in the third instar, growth is continuous, except at the time of molting, until the larva is full grown. The weight then remains approximately constant until the beginning of metamorphosis. When the resting stage occurs in the second instar, growth ceases temporarily in the second instar for a period averaging approximately 5 weeks. After the larva has become full grown, metamorphosis begins immediately.

4. Larvae reared at 20° attain a lower maximum weight but require a longer growth period than those reared at 25° C., while

adults reared from larvae at 20° are smaller than those reared from larvae at 25°. At both temperatures there is a definite sex difference in weight, the female adults averaging 40–50 milligrams heavier than the males.

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INSECT DEVELOPMENT. V. QUALITATIVE STUDIES ON THE FATTY ACIDS FROM GRASSHOPPER EGGS

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DURING the course of a quantitative study of the fatty acids from the eggs of *Melanoplus differentialis*, one of the more common grasshoppers, a single analysis was made of the eggs of another grasshopper, *Chortophaga viridifasciata* (Slifer, 1930). The fatty acids obtained from the eggs of this last-named species, it was found, solidified, at room temperature, immediately after isolation, while those from *Melanoplus differentialis* remained liquid, closely resembling olive oil in appearance and consistency.

In this region a few species of Acrididae—of which *Chortophaga viridifasciata* is one—spend the winter as nymphs, mature in the spring, and a little later deposit their eggs. Such eggs ordinarily hatch during the summer and so are never exposed to low temperatures. *Melanoplus differentialis*, on the other hand, as do the majority of grasshoppers, lays its eggs in the late summer and fall. These remain in the ground over winter and in consequence are normally subjected to severe and repeated freezings. Since the fats in the egg are evidently of prime importance as a source of food, the possibility suggested itself that some correlation might exist between the environmental conditions under which the egg normally develops and the character of the fats stored in it. With this problem in mind a number of different species of grasshoppers were collected and certain of the properties of the fatty acids from their eggs determined.

In addition to this, analyses were made of the eggs of *Melanoplus differentialis* at selected intervals during incubation, with a view to detecting changes—should such take place—in the degree of saturation of the fats as growth proceeded. Eaves, in 1910, reported the occurrence of alterations of this nature in the developing hen's egg.

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METHODS AND RESULTS

The eggs used in the series of experiments about to be described were laid by female grasshoppers which were confined in cages in the laboratory and furnished daily with a generous supply of fresh grasses, weeds, and lettuce.

Since the availability of a fat for nutritional purposes depends largely upon the number of double bonds present (Leathes and Raper, 1925), and since there is frequently a close relationship between the number of double bonds and the melting-point, it was believed that the iodine absorption numbers of the various fatty acids might give quantitative data which would be of some value in comparing the different fats with one another.

For the isolation of the fatty acids Belin's (1926) modification of the original saponification method of Kumagawa and Suto was employed, a few minor changes being introduced. The eggs—one hundred or more in number—were removed from the pod in which they were laid and washed in distilled water until free from all foreign matter. They were then heated with 25 cc. of 40 per cent potassium hydroxide and a few cubic centimeters of redistilled alcohol under a reflux condenser for $1\frac{1}{2}$–2 hours. At the end of this time the material was removed to a separatory funnel, and enough alcohol added to give a 50 per cent solution. After cooling to room temperature, this was shaken three times with redistilled petroleum ether; and the several extracts containing any sterols, pigments, etc., which might be present, were removed. Sufficient hydrochloric acid was added to more than neutralize the alkali, and the whole again allowed to cool. The fatty acids were then shaken out with fresh petroleum ether and the extract washed repeatedly with distilled water to remove traces of hydrochloric acid. The petroleum ether containing the fatty acids was transferred to a clean beaker and enough anhydrous sodium sulphate added to absorb the remaining water. The solution was finally filtered into a tared 30 cc. beaker and evaporated to dryness in a current of carbon dioxide. The beaker with the fatty acids was weighed at once, the acids dissolved in chloroform and transferred quantitatively to an iodine titration flask. The Hanus method, as described in *Official and Tentative Methods of Analysis*

of the Association of Official Agricultural Chemists (1925), was then followed for the determination of the iodine number.

The accuracy of the iodine determinations was checked by experiments with chemically pure oleic acid in amounts equivalent to those usually secured from the grasshopper eggs. The iodine number for oleic acid is 90.07, while the mean obtained with nineteen experiments of this sort was 90.3 ± 1.8.

As may be seen by examining Table I, the iodine numbers for the fatty acids from *Melanoplus differentialis* eggs indicate the presence of a large number of double bonds; and there is no significant change in the degree of saturation during the developmental period, regardless of the presence or the absence of a diapause.[1] This suggests that the embryo is able to use, indiscriminately, saturated or unsaturated fats. However, the fats present when the egg is laid may *all* be more or less highly unsaturated and so make the selection of certain ones unnecessary. It is also possible that the iodine number of the fats in the yolk may fall and that of the fats in the embryo rise, in such a manner as to cancel one another. The figures for the fatty acids from eggs exposed to low temperatures tend to run a bit higher than those from eggs kept at 25° C., but the difference is scarcely significant.

The iodine numbers of the fatty acids from the freshly laid eggs of six other species of Acrididae were determined, with the results shown in Table II. These acids, like those from *Melanoplus differentialis* eggs, are also evidently highly unsaturated, their iodine numbers being of the same general order of magnitude.

Despite the similarity in iodine numbers, the fatty acids from the seven species of grasshoppers mentioned above fall into two distinct classes—those which are largely solid at room temperature and those which are liquid under such conditions. The acids from the eggs of *Arphia sulphurea*, *Chortophaga viridifasciata*, and *Hippiscus apiculatus* belong to the first group; while those from *Dissosteira carolina*, *Melanoplus differentialis*, *M. femur-rubrum*, and *M. walshii*

[1] "On peut désigner sous le nom de *diapauses* (διάπαυσις, repos, interruption de travail) ces périodes d'arrêt dans le développement ontogénétique d'un animal, depuis la fécondation de l'œuf jusqu'à l'âge adulte, et distinguer des diapauses embryonnaires, des diapauses larvaires et des diapauses nymphales" (Henneguy, 1904).

TABLE I

IODINE NUMBERS OF FATTY ACIDS FROM EGGS OF *Melanoplus differentialis*

Exp. No.	Iodine No.	Mean
One-Day Old		
1	135.4	
2	135.2	
3	126.9	
4	132.9	
5	132.8	
6	132.5	
7	132.9	
8	132.2	
9	128.6	
10	131.5	
11	128.1	
12	128.5	131.5
Early Diapause (30–40 Days at 25°)		
13	120.3	
14	134.4	
15	141.2	
16	136.5	
17	123.9	
18	140.0	
19	143.7	
20	142.0	
21	130.5	
22	124.9	
23	130.6	
24	133.4	133.5
Postrevolution, Following Extended Diapause at 25° C.		
25	134.9	
26	104.5	
27	132.0	123.8
Outdoors for 4 Months or More, Then Analyzed		
28	140.5	
29	145.9	
30	140.0	
31	120.0	
32	143.5	
33	138.8	
34	145.3	
35	146.9	
36	136.3	
37	140.3	
38	151.8	
39	133.0	140.2

TABLE I—*Continued*

Exp. No.	Iodine No.	Mean
Hatching (Outdoors 4 Months or More, Then 25° C.)		
40	135.4	
41	143.1	
42	126.7	
43	129.7	
44	129.8	132.9
Hatching, without a Diapause		
45	141.2	
46	132.5	
47	148.3	140.7

belong to the latter. This distinction assumes greater interest when it is recalled that the first three of these species hibernate as nymphs and lay eggs which develop during warm weather; while the other four lay eggs in the fall which are exposed normally to low temperatures during the winter and hatch the following spring.

TABLE II

IODINE NUMBERS OF FATTY ACIDS FROM DAY-OLD GRASSHOPPER EGGS

Species	Iodine No.
Dissosteira carolina	128.4
Melanoplus femur-rubrum	133.8
Chortophaga viridifasciata	134.0
Hippiscus apiculatus	142.0
Melanoplus walshii	151.1
Arphia sulphurea	166.9

The differences displayed by the fats from the two groups of eggs were so marked that it was decided to make melting-point determinations on all available species. For this purpose the melted fatty acids, immediately after isolation, were drawn up into small capillary tubes, which were sealed and placed in corked vials. These vials were frozen into a block of ice in an electric refrigerator and kept there for at least 24 hours before the melting-points were determined. Since the fatty acids from the eggs are mixtures rather than single chemical compounds, the melting-points are not sharply defined.

It is relatively easy to determine the point at which fusion is complete, but the temperature at which it begins is difficult to ascertain with any exactness. In all species a trace—sometimes barely perceptible, sometimes quite distinct—is liquid at 1° C. As the temperature is raised, little alteration is noticeable until 20° C. is reached. At this point the crystals of fatty acid in samples from *Melanoplus differentialis* eggs begin to disappear rapidly. Between 22° and 24° C. the greater portion of the acids melt in *Arphia xanthoptera*, *Dissosteira carolina*, *Encoptolophus sordidus*, *Hippiscus rugosus*, *Melanoplus bivittatus*, and *M. femur-rubrum*. Fusion is most rapid at 27° C. in specimens from *Spharagemon bolli* eggs; while there is almost no evidence of change in *Arphia sulphurea*, *Chortophaga viridifasciata*, and *Hippiscus apiculatus* fatty acids until 30° C. is reached and passed. The points at which fusion is complete are shown in Table III.

TABLE III

POINTS OF COMPLETE FUSION OF FATTY ACIDS FROM DAY-OLD GRASSHOPPER EGGS*

Species	Fusion Complete
Melanoplus bivittatus	25.5° C.
M. differentialis	26.2°
Encoptolophus sordidus	26.2°
Melanoplus femur-rubrum	26.6°
Dissosteira carolina	28.4°
Arphia xanthoptera	28.5°
Hippiscus rugosus	29.0°
Spharagemon bolli	30.5°
Hippiscus apiculatus	37.0°
Chortophaga viridifasciata	39.4°
Arphia sulphurea	39.5°

* The fatty acids from day-old eggs of *Melanoplus walshii* are also liquid at room temperature, but melting-point data were not obtained.

Of the eleven species studied, *Arphia sulphurea*, *Chortophaga viridifasciata*, and *Hippiscus apiculatus* lay eggs in the spring which hatch that same summer. All the rest lay eggs in the fall which, under normal conditions, are exposed to low winter temperatures before completing their development. It is especially interesting to compare *Arphia sulphurea* with *A. xanthoptera* and *Hippiscus api-*

culatus with *H. rugosus*. In each of these cases two species within a genus lay their eggs at different seasons, and the fatty acids from their eggs show a marked tendency to melt over ranges particularly suitable to the specific conditions under which embryonic development occurs.

That a close correlation frequently exists between the properties of the fat synthesized by a plant or animal and the temperature under which it is laid down has been known for a long time. Thus, to quote Leathes and Raper (1925):

> There is reason to believe that all the fats found in nature are fluid at the temperature at which they are normally formed in the animal or plant. The fats found in cold-blooded animals, or in plants growing in temperate or cold climates, are therefore always unsaturated to a considerable extent, since the unsaturated acids form triglycerides with low melting points. On the other hand, the saturated fats occurring in nature are only found to any marked extent in warm-blooded animals or in tropical plants, since the presence of saturated fatty acids, especially those containing 16 or 18 carbon atoms, gives rise to fats which are solid at the ordinary temperature.

Leathes and Raper make the interesting suggestion that a simple explanation of these facts would be that "fats are formed in nature by a series of reactions, the end points of which vary according to the temperature at which they occur." They would consider, then, the unsaturated fats intermediate products in the synthesis of the more saturated fats. At high temperatures the reactions reach completion, but at lower temperatures the end-product is one of the intermediate compounds.

It is difficult to see just how the facts detailed above, concerning the grasshopper eggs, could be brought into line with this suggestion. The acids are decidedly different in character; yet all were produced by females exposed to the same laboratory temperatures. Neither will an appeal to differences in the foods consumed by the animals settle the question, for all were furnished with the same diet and ate it with apparent equal readiness. Moreover, it is extremely likely that the greater part of the fat deposited in the eggs is formed originally from the plant carbohydrates eaten by the insect, since the amount of fat present in such food is quite small, while starches, sugars, etc., are relatively abundant.

The ability to manufacture fats of a certain type appears, then, to be a species characteristic associated with a particular mode of life; and in the present case, at least, it seems wiser not to attempt to explain this relationship on the basis of the action of the environment during the synthetic process.

SUMMARY

1. There is no significant change during the period of incubation in the degree of saturation (as measured by the iodine number) of the fatty acids present in the eggs of *Melanoplus differentialis*.

2. The iodine numbers of the fatty acids from *Melanoplus differentialis* eggs developing with a diapause differ little, at hatching, from those developing without it.

3. No significant difference was found between the iodine numbers of eggs in which the diapause had been broken by exposure to low temperatures and the iodine numbers of eggs not so treated and, in consequence, still in the diapause.

4. The fatty acids from the newly laid eggs of seven species of grasshoppers—*Arphia sulphurea, Chortophaga viridifasciata, Dissosteira carolina, Hippiscus apiculatus, Melanoplus differentialis, M. femur-rubrum, and M. walshii*—contain numerous unsaturated linkages, as is evidenced by their high iodine numbers.

5. The fatty acids from the day-old eggs of the twelve species of Acrididae studied may be separated into two groups: (1) those which are largely liquid at room temperature and (2) those which are solid. Under normal outdoor conditions the nine species with the more liquid fats lay eggs in the fall which undergo a long exposure to cold weather before hatching the following spring. The three species which lay eggs in the spring which hatch that same summer, on the other hand, produce mixtures of fatty acids with considerably higher melting-points. This was found to apply (in two instances) even to species within the same genus, when those species were accustomed, in nature, to laying their eggs at such seasons as would expose them, in the several cases, to radically different temperatures during development.

6. The conclusion is reached that factors other than environ-

mental play a leading rôle in determining the type of fat laid down in the eggs of Acrididae.

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THE EFFECT OF X-RAYS ON THE GASEOUS METABOLISM OF A CLADOCERAN

(One figure)

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INTRODUCTION

WHEN single cells, groups of cells, or entire organisms are subjected to X-rays of sufficient strength, there is produced temporary or permanent injury to the living matter. In studying more precisely the biological effects of X-rays, it becomes necessary that the internal changes produced be measured in terms of some definite set of chemical or physical transformations. A group of workers has already discussed the nature of curves obtained by plotting the intensity of X-ray exposure as a function of the rate of killing of the living matter under investigation. Such graphic representations have enabled the investigators in the field to advance some concepts pertaining to the nature of the action of X-rays on living matter with respect to the number of quanta of energy employed and the effects produced. Since the physical and chemical changes produced in a cell must necessarily be accompanied by an alteration in metabolism, it is evident that a study of the rate of oxygen consumption or carbon dioxide production furnishes us with a more direct criterion in studying the quantitative effect of X-rays on the cell functioning. The present paper is primarily concerned with a study of the effects of X-rays on *Simocephalus vetulus*, with special reference as to how they alter the gaseous metabolism of the organism as judged by the rate of oxygen consumption and the energy relations involved.

It is a well-known fact that different kinds of cells and tissues vary in sensitivity to X-ray exposure. Sex cells, for example, possess marked susceptibility to this radiant energy. They may be killed or injured by certain intensities of X-radiation which produce no meas-

urable effect on the somatic cells. *Simocephalus vetulus*, however, is a fairly simple organism, measuring about 2 mm. in length and less than 0.5 mm. in width at sexual maturity. Its structures and organ systems are transparent even under the low power of the microscope. Subjecting such an organism to X-rays and studying the effect produced is of no less interest or importance than when a single cell or a group of cells is employed. For, whereas a single cell or group of similar cells enables us to follow the velocity of changes produced in living matter when these cells are all equally sensitive to the radiant energy, a study of a minute and transparent organism such as *S. vetulus* gives us a picture of the activities of varieties of tissues and structures. The measurement of the changes produced in such an organism represents the average of activities of many parts, still in contact with the various mechanisms which control some of the vital processes. The method employed in this study enables us to measure very minute quantities of oxygen utilized by the organism at definite and precise stages in its development and under well-controlled laboratory conditions. The regulation of such factors are of importance in studies of this type. This work was done in the laboratories of the Department of Zoölogy and the Department of Radiology, Syracuse University. We wish to express our indebtedness to Dr. W. M. Smallwood, director of the zoölogical laboratories, and his entire staff, for the many courtesies we received in the course of our research.

APPARATUS AND METHODS

The animals subjected to experimentation were obtained from a pond in the vicinity of Poughkeepsie, New York, and represent individuals descended from a single common mother brought to the laboratory November 24, 1930. They were reared in the laboratory parthenogenetically for approximately ten months before subjecting them to experimentation in this study. The cultural method employed in rearing genotypically identical individuals, as well as the method employed in measuring the minute quantities of oxygen consumed by the animals, has already been described in previous papers (Obreshkove and Banta, 1930; Obreshkove, 1930). The animals were reared before and after X-raying, in individual bottles containing standard amount of the culture medium, and were kept in a water

bath which was set at 25° C. ±0.01 C. The apparatus employed for the measurement of the rate of oxygen consumed by the animals was inserted in a Freas water bath with a thermostat control also set at 25° C. The temperature in this bath was constant to ±0.005° C.

The animals were subjected to X-radiation produced at 30 K.V. A Coolidge X-ray tube was used, containing a molybdenum target. This tube was operated on a single-phase circuit. Since the target was water cooled, the tube acted as its own rectifier, and hence produced a pulsating beam with 60 pulses per second (half-wave rectification). Under the foregoing conditions, the shortest length of the X-rays in the emission was approximately 0.41 Angstrom units (0.41×10^{-8} cm.). However, with the molybdenum target operating at 30 K.V., the greatest portion of the emitted energy comes forth as the molybdenum $K\alpha$ radiation, which has an average wave-length of approximately 0.712 Angstrom units (A). The radiation, therefore, is relatively soft and is in the intermediate range. The irradiation of the organisms was continued for 1 hour in each experiment, in a beam of measured intensity. No filter was employed in this set of experiments. The intensity was measured with an ionization electrometer kindly furnished by the Victoreen Instrument Company.

A few words on the course of the life-cycle of *Simocephalus vetulus* may be of help in interpreting the results to be presented. This species, when reared in the laboratory under favorable conditions, produces eggs which develop without being fertilized. The eggs are deposited in a cavity lying just above the dorsal part of the valves inclosing the animal and the upper side of the body. This is the brood chamber. The number of eggs deposited varies with the laboratory conditions, but usually from 10 to 12 eggs are laid when the animals are kept at 25° C. and in standard food supply. Since the individuals are transparent under the microscope, the subsequent history of the eggs is easily observed. They begin a rapid segmentation and are nourished in the course of their development by the yolk contained in the eggs themselves, and likewise by a fluid in the brood chamber secreted by glandular cells at the upper portion of the chamber. The eggs are transformed into fully formed young in about 48 hours after their deposition, are released then, and become free-swimming individuals. The young, immediately after their release, measure

about 0.5 mm. in body length. No other appreciable change in the length of the individual is observed till after the first ecdysis takes place some 48 hours later. Each animal undergoes four such sheddings of the carapace before sexual maturity, each ecdysis marking a new instar in the development of the individual. The young become sexually mature in about 6 days after their release, and at that time deposit their first clutch of young.

In studying the effects of X-rays on the gaseous metabolism, the young were subjected to X-radiation of known intensities immediately after their release, but the rate of oxygen consumption was measured 6 days later at a time when untreated animals normally reach sexual maturity. The following intensities were employed: 164, 251, 327, and 404 *r*-units per minute of irradiation produced at 30 K.V.; and in each case the exposure was 1 hour. In another set of experiments, and in connection with another problem, we employed a more powerful 300 K.V. three-phase constant-potential X-ray apparatus. The primary aim of this paper, however, is to report the effect of relatively soft X-rays and of intermediate range of the type employed in this particular work.

OBSERVATIONS

Oxygen consumption after 164 r-units per minute irradiation.—In subjecting the individuals to a beam of X-rays of known intensity, the animals were placed in a depression slide covered with just enough of the culture medium to allow free swimming of the individuals without altering very much the distance from the target to the individual animal. This procedure enabled us to better control the intensity factor. It was observed that an intensity above 404 *r*-units per minute and 1 hour exposure reduced the rate of oxygen consumption until it was altogether too slow to permit an accurate measurement. On the other hand, an intensity of 164 *r*-units per minute represents a dose lying just above the threshold value of effectiveness.

Table I gives the results obtained with an intensity of irradiation consisting of 164 *r*-units per minute and 1 hour exposure. The table shows that *Simocephalus vetulus*, X-rayed immediately after their release, consume oxygen when 6 days old at the rate of 56.9×10^{-7} cc.

per individual per minute. On the other hand, untreated individuals (Table II) at the same age utilize oxygen at the rate of 68×10^{-7} cc. per individual per minute. Columns 1 and 2 in these and in other similar tables which follow, give the average of the body lengths and also the average of the cube of the body lengths for each group of four individuals tested. In determining the average cube of the body

TABLE I

OXYGEN CONSUMPTION OF *Simocephalus vetulus* FEMALES 6 DAYS OLD, IRRADIATED FOR 1 HOUR IMMEDIATELY AFTER THEIR RELEASE FROM THE BROOD CHAMBER. DOSAGE, 164 *r*-UNITS PER MINUTE AT 30 K.V.

(Individuals were tested in groups of four)

AVERAGE LENGTH IN MILLIMETERS (1)	AVERAGE CUBE OF BODY LENGTH (2)	O_2 CONSUMPTION, CC. $\times 10^{-7}$	
		Per Group Tested per Hour (3)	Per Individual per Minute (4)
1.77	5.55	9,858	41.0
1.77	5.55	9,858	41.0
1.85	6.33	12,322	51.3
1.80	5.83	16,845	70.1
1.85	6.33	14,787	61.6
1.66	4.57	9,188	38.2
1.75	5.36	14,931	62.2
1.80	5.83	16,462	68.6
1.75	5.36	14,548	60.6
1.66	4.57	11,868	49.4
1.70	4.91	13,144	54.7
1.85	6.33	12,323	51.3
1.70	4.91	16,430	68.4
1.85	6.33	12,333	51.3
(Av.) 1.76	5.50	.....	56.9

lengths for each group tested, and also for each entire series of experiments under one type of conditions, the length of each individual was cubed separately, and from this the average for the entire group under consideration was determined. The average body length of the untreated animals was found to be 1.71 mm. The individuals which were X-rayed with a dose consisting of 164 *r*-units per minute for 1 hour at 30 K.V., measured on the average 1.76 mm. in body length. This slight difference in the body length of the two groups of individuals is, however, of no statistical value. It is evident from the inspection of this and other data presented in Tables I and II

that, although the X-rayed individuals happened to be, on the average, slightly larger, their rate of oxygen consumption is lower per individual per minute. The tables give further data involved in the comparisons.

Oxygen consumption after irradiation with higher intensities.—Turning now to the results obtained with individuals which have been X-rayed with an intensity consisting of 251 *r*-units per minute and of 1 hour duration, we observe not only that there is a further

TABLE II

OXYGEN CONSUMPTION OF UNTREATED *Simocephalus vetulus* FEMALES AT SEXUAL MATURITY

No. of Individuals in Chamber	Average Length in Millimeters (1)	Average Cube of Body Length (2)	O_2 Consumption, Cc. $\times 10^{-7}$	
			Per Group Tested per Hour (3)	Per Individual per Minute (4)
3	1.93	7.18	11,485	63.8
3	1.59	4.03	13,555	75.3
3	1.59	4.03	12,733	70.7
4	1.75	5.41	20,290	84.5
4	1.71	5.00	13,782	57.4
3	1.68	4.74	11,090	60.6
4	1.75	5.41	16,270	67.7
3	1.68	4.74	12,528	69.6
4	1.75	5.41	16,844	70.1
4	1.71	5.00	14,547	61.6
Average	1.71	5.11	.....	68.0

decrease in the rate of oxygen consumption with this higher intensity but that there is shown also a retardation in the growth of the individuals. The results are given in Table III. Sixteen groups of individuals with four animals in each group were tested. The average body length for the entire group tested is shown to be 1.64 mm. On the other hand, as pointed out earlier, individuals subjected to 164 *r*-units per minute X-radiation and 1 hour exposure, averaged 1.76 mm. in body length; thus the individuals subjected to the stronger intensity of irradiation showed an average decrease of 0.12 mm. in body length per individual.

Untreated individuals were shown to consume oxygen at the rate of 68×10^{-7} cc. per individual per minute (Table II). Animals which were X-rayed with an intensity consisting of 164 *r*-units per minute

showed a rate 56.9×10^{-7} cc. per individual per minute; while those individuals which were treated with the still higher intensity, namely, 251 *r*-units per minute, showed a still lower rate of oxygen consumption, namely, 42.4×10^{-7} cc. per minute per individual.

It is evident from the comparison presented above that whatever may be the other effects of X-rays on *Simocephalus vetulus*, on the

TABLE III

OXYGEN CONSUMPTION OF *Simocephalus vetulus* FEMALES 6 DAYS OLD, IRRADIATED FOR 1 HOUR IMMEDIATELY AFTER THEIR RELEASE FROM THE BROOD CHAMBER. DOSAGE, 251 *r*-UNITS PER MINUTE AT 30 K.V.

(Individuals were tested in groups of four)

AVERAGE LENGTH IN MILLIMETERS (1)	AVERAGE CUBE OF BODY LENGTH (2)	O_2 CONSUMPTION, CC. $\times 10^{-7}$	
		Per Group Tested per Hour (3)	Per Individual per Minute (4)
1.77	5.58	13,016	54.2
1.77	5.58	12,634	52.6
1.72	5.09	8,422	35.1
1.64	4.45	10,269	42.7
1.56	3.88	11,485	47.8
1.64	4.45	10,269	42.7
1.56	3.88	8,040	33.5
1.69	5.05	13,144	54.7
1.69	5.05	9,858	41.0
1.62	4.28	8,422	35.0
1.56	3.88	11,485	47.8
1.66	4.66	8,626	35.9
1.56	3.88	8,040	33.5
1.66	4.25	11,485	47.8
1.66	4.25	1,072	44.6
1.66	4.66	1,848	30.8
(Av.) 1.64	4.55	.....	42.4

organism as a whole or on the cells of which it is composed, with the increase of dosage of irradiation, there is produced a pronounced decrease in the rate of gaseous metabolism of the organism. Likewise, increasing the intensity of irradiation produces a proportional decrease in the rate of growth of the individuals. With still higher intensities than heretofore reported, this inverse relation between the intensity of irradiation and the rate of oxygen consumption and of growth is retained. Tables IV and V give the results obtained with 327 and 404 *r*-units per minute irradiation. As in all other experi-

ments, the exposure was 1 hour at 30 K.V. The average body length of the individuals subjected to the weaker X-ray dose is shown to be

TABLE IV

OXYGEN CONSUMPTION OF *Simocephalus vetulus* FEMALES 6 DAYS OLD, IRRADIATED FOR 1 HOUR IMMEDIATELY AFTER THEIR RELEASE FROM THE BROOD CHAMBER. DOSAGE, 327 *r*-UNITS PER MINUTE AT 30 K.V.

(Individuals were tested in groups of four)

AVERAGE LENGTH IN MILLIMETERS (1)	AVERAGE CUBE OF BODY LENGTH (2)	O_2 CONSUMPTION, CC. $\times 10^{-7}$	
		PER GROUP TESTED PER HOUR (3)	PER INDIVIDUAL PER MINUTE (4)
1.48	3.34	6,891	28.7
1.47	3.18	7,394	30.8
1.48	3.34	6,508	27.1
1.47	3.18	7,394	30.8
1.47	3.18	6,891	28.7
1.47	3.18	7,804	32.5
1.47	3.18	6,508	27.1
1.51	3.44	5,743	23.9
1.51	3.44	5,750	23.9
1.51	3.44	6,508	27.1
(Av.) 1.49	3.35	.....	28.1

TABLE V

OXYGEN CONSUMPTION OF *Simocephalus vetulus* FEMALES 6 DAYS OLD, IRRADIATED FOR 1 HOUR IMMEDIATELY AFTER THEIR RELEASE FROM THE BROOD CHAMBER. DOSAGE, 404 *r*-UNITS PER MINUTE AT 30 K.V.

(Individuals were tested in groups of four)

AVERAGE LENGTH IN MILLIMETERS (1)	AVERAGE CUBE OF BODY LENGTH (2)	O_2 CONSUMPTION, CC. $\times 10^{-7}$	
		PER GROUP TESTED PER HOUR (3)	PER INDIVIDUAL PER MINUTE (4)
1.43	2.99	4,518	18.8
1.43	2.99	4,107	17.1
1.43	2.99	4,929	20.5
1.35	2.52	3,828	15.9
1.48	3.27	5,359	22.3
1.35	2.52	5,359	22.3
1.48	3.27	4,976	20.7
1.43	2.98	5,750	23.9
1.48	3.27	3,828	15.9
1.44	2.98	6,161	25.6
1.43	2.99	5,339	22.2
1.43	2.99	4,518	18.8
(Av.) 1.43	3.03	.....	20.3

1.49 mm. and that of the animals exposed to the stronger intensity of X-radiation was found to be 1.43 mm. This represents a decrease of 0.22 mm. and 0.28 mm., respectively, over the average body length of the untreated *S. vetulus*. An inspection of the tables, particularly the figures in column 4 show also that these higher intensities are more effective in producing changes in the organisms. The individual readings are shown here to be more nearly constant, in that there is observed less variation in the consecutive readings obtained under the same conditions.

For a test of the accuracy of the apparatus and to check the effectiveness of the X-rays, often the same group of individuals after sufficient rest was placed in the respirometer for a second and third time. Such experiments showed surprising uniformity in results. In many cases, no change whatever was observed in the rate of oxygen consumption from the previous records made for the same group. It is, therefore, thought that the experimental conditions were well controlled and that the method can be satisfactorily employed for a study of the gaseous metabolism of these very minute organisms.

Rate of oxygen consumption, size considered.—Simocephalus vetulus is too small to permit accurate weighing of a single individual or of a small group of individuals. It is evident, however, that in view of the relation which we have pointed out to exist between the intensity of radiation and the rate of oxygen consumption per individual per minute, for a more critical analysis of the influence of X-rays on the gaseous metabolism of the individual, it is necessary to make a comparative study on the basis of a unit of protoplasm. For this comparative study we utilized the rule that the volumes of two similar but irregular bodies vary as the cube of their body lengths. Table VI gives summary figures for a comparison of the rate of oxygen consumption at the various intensities. Dividing the average rate of oxygen consumption per individual per minute for each series of experiments by the corresponding average cube of the body lengths for that series, we have obtained the rate of oxygen consumption per cubic millimeter of volume for each intensity of irradiation. On the basis of this calculation, the table reveals that the untreated individuals utilize oxygen at the rate of 13.3×10^{-7} cc. per cubic millimeter of volume. For individuals which have been subjected to a dosage con-

sisting of 164 *r*-units per minute for 1 hour at 30 K.V., the rate becomes 10.3×10^{-7} cc. for the same unit of protoplasm. If the rate of oxygen consumption of the untreated individuals be denoted by 100 per cent, then the rate of the X-rayed animals with this intensity becomes 77.4 per cent that of the untreated. This decrease in the rate of oxygen consumption continues with the increase of the dosage of X-radiation. With 251 *r*-units per minute irradiation, the rate becomes 70.0 per cent; with 404 *r*-units it falls to 50.4 per cent. Table VI gives further data involved in the comparisons.

TABLE VI

SUMMARY TABLE ON THE RATE OF OXYGEN CONSUMPTION BY *Simocephalus vetulus* FEMALES AFTER IRRADIATION

	ROENTGEN UNITS				
	0	164	251	327	404
Average body length (mm.)	1.71	1.76	1.64	1.49	1.43
Average cube of body length	5.11	5.50	4.55	3.35	3.03
Oxygen consumption per individual per minute, cc. $\times 10^{-7}$	68.0	56.9	42.4	28.1	20.3
Oxygen consumption per cubic millimeter of body volume per minute, cc. $\times 10^{-7}$	13.3	10.3	9.3	8.4	6.7
Rate of oxygen consumption compared with untreated individuals (per cent)	100.0	77.4	70.0	63.1	50.4

Plotting the rate of oxygen consumption expressed in percentage (Table VI) as a function of the intensity of X-radiation, we find that this relation is linear. This is shown in Figure 1. The graphic representation characterizes the steady rate of decrease of oxygen consumption with the increase of irradiation.

DISCUSSION

In studying more precisely the biological effect of X-rays under various conditions, it becomes necessary that the physiological effect produced be measured in terms of some definite set of internal changes. A study of the rate of oxygen consumption of *Simocephalus vetulus* after X-radiation serves as a direct criterion in studying the degree of internal disturbances produced. This method of study has an advantage over the death-rate graphic representations employed by a number of workers. It is evident from the nature of the experimental work presented in this paper that we are not studying the

effect of X-rays on cells of equal sensitivity but that we are, rather, recording the effect on varieties of tissues and structures. When an entire organism is subjected to the influence of X-rays, a determina-

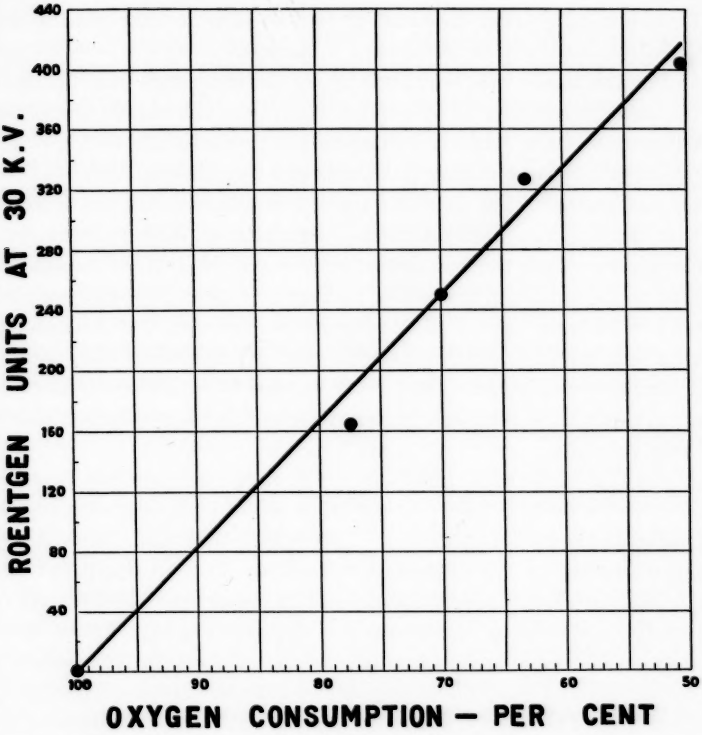

FIG. 1.—Relation between the X-ray dosage and the oxygen consumption per unit of volume of *Simocephalus vetulus*. The dosage in *r*-units per minute is plotted as a function of the rate of oxygen consumption. The latter is expressed as the percentage of the rate of oxygen consumption of the untreated females of the same age.

tion of the rate of oxygen consumption of such an individual represents the average rate of the gaseous metabolism of the varieties of tissues and structures of which the organism is composed. Each tissue of the body responds to the X-rays in accordance with its own degree of susceptibility to the radiant energy. The intensities of X-

radiation employed in this work varied in range from that just above the threshold value of effectiveness to an intensity which proved to be very strongly effective. Since each intensity of X-radiation produced a definite set of internal changes, judging from the rate of oxygen consumption, the work presented here permits us to make a quantitative study of the effects of X-rays.

The metabolic rate is fairly high in the untreated individuals. If this rate be represented by 100 per cent, then the rate in individuals which have been subjected to an intensity consisting of 164 *r*-units per minute for 1 hour at 30 K.V., becomes 77.4 per cent that of the untreated animals. The rate of oxygen consumption further decreases with the increase of the intensity of irradiation. With 251 *r*-units per minute exposure, the rate becomes 70.0 per cent. With the application of 327 *r*-units per minute, and again 1 hour exposure, the rate falls to 63.1 per cent; and 404 *r*-units per minute irradiation brings a further decrease in the metabolic rate of the individuals, namely, it becomes 50.4 per cent that of the normal rate. These comparisons are computed on the basis of individuals of the same age per unit of body volume.

The intensities of X-radiation of the range employed in these experiments proved lethal to the ovarian cells and the eggs already deposited in the brood chamber. It is evident, therefore, that the oxygen consumed by the untreated individuals given in this paper cannot be ascribed to the organisms alone; a fair amount of it will represent that utilized by the ovary and the growing eggs in the brood chamber. It is fair to assume that the rate of gaseous metabolism of the untreated *Simocephalus vetulus* is somewhat lower than actually computed. The observed decrease in the rate of metabolism of the X-rayed individuals, however, cannot be attributed entirely to their sterile condition. If a comparative study is made, for example, based on the individuals exposed to a dose consisting of 164 *r*-units per minute and denoting their rate of oxygen consumption as 100 per cent, there is observed the same degree of decrease in the rate of gaseous metabolism with the increase of intensity of irradiation. It is obvious that the X-rays of the type and range employed in this study act in a definite and orderly fashion in producing internal changes in living matter. The relation which exists between the

intensity of X-radiation and the internal changes produced is further substantiated by a study of the intensity of irradiation and the rate of growth of the individuals. It will be observed from the other data presented that, with the increase of the strength of the energy applied, there is a definite and proportionate retardation of the rate of growth of the individuals. It is interesting to note here that Wels (1924), working with bacteria, yeast, and muscle cells did not observe any change in the respiratory rate after radiation. Bersa (1927) reports a decrease in respiration in irradiated beans. On the other hand, Redfield and Bright (1922) hold that there is no relation between the rate of CO_2 production and growth in seeds.

In *Simocephalus vetulus*, X-radiation does not produce only a change in the rate of the gaseous metabolism and in the rate of growth, but it also produces prominent physical changes. These changes involve the upper portion of the brood pouch. This portion of the animal after exposure to X-rays is altered from a convex to a concave structure in outline. This condition will be reproduced and described in more detail in another paper. It suffices to say here that the produced invagination increases in prominence with the increase of the intensity of X-radiation and elapse of time. In this study 240 individuals were irradiated. Without a single exception a marked invagination was produced over the dorsal portion of the brood pouch.

The production of such a physical change as described above is of interest from several points of view. First of all, it must be recalled that the animals were subjected to X-rays immediately after their release from the brood pouch. At this stage in their development they measure about 0.5 mm. in body length. In their subsequent growth, from the time of their release up to the time which corresponds to sexual maturity in the untreated individuals, they have shed their carapace four times and have reached about 2 mm. in body length. With each succeeding ecdysis, the invagination becomes more and more prominent, thus suggesting that the physical change once produced is retained in the subsequent growth of the cells involved. The physical change under discussion is of interest, also, because it involves a region in the brood pouch which is lined with very active cells engaged in producing a fluid which is poured

into the brood chamber. This fluid furnishes nourishment for the developing eggs and embryos.

It is very possible that other less active somatic cells of *Simocephalus vetulus* are affected in like manner by X-rays to a lesser degree. These other cells are more firmly held by connective and muscular tissues, and for this reason it is very probable that the distortion in outline of the cells involved becomes less prominent. It has been suggested that the physical changes described in the very active glandular cells of the brood chamber are the result of changes in cell permeability. This supposition appears very logical; and if true, it raises a number of very interesting questions, namely, the relation which may exist between injury, gaseous metabolism, and permeability. In the opinion of some writers (Osterhout, 1912), permeability is a relatively fixed property of the cell and changes as a result of injury. If the physical change described, represented by an invagination extending from end to end over the dorsal portion of the brood pouch, is the result of changes in cell permeability, it seems obvious from our results on the rate of oxygen consumption after treatment with X-rays that changes in cell permeability may control cellular metabolism.

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SUMMARY

1. The rate of oxygen consumption of *Simocephalus vetulus* was studied after the organism had been subjected to various intensities of X-radiation.

2. The individuals were subjected to X-rays immediately after their release from the brood chamber; the rate of oxygen consumption was determined 6 days later at a time when untreated individuals became sexually mature.

3. The rate of gaseous metabolism of X-rayed individuals was compared with that of untreated animals, size considered; and the effect of the X-rays and the energy relations involved were studied.

4. The metabolic rate is fairly high in untreated individuals. If

this rate is denoted by 100 per cent, then with an intensity of irradiation consisting of 164 *r*-units per minute and 1 hour exposure at 30 K.V., the rate becomes 77.4 per cent of the normal rate.

5. With a further increase in the intensity of X-radiation, there is observed a further decrease in the gaseous metabolism. It becomes 70.0 per cent when the animals are exposed to irradiation of 251 *r*-units per minute and all other factors kept the same. With 327 *r*-units per minute irradiation, the rate of oxygen consumption falls to 63.1 per cent, and with 404 *r*-units per minute it is 50.4 per cent that of the untreated animals.

6. When the rate of oxygen consumption is plotted as a function of the intensity of X-radiation, the relation is linear.

7. With the increase in the intensity of X-radiation there is likewise observed a definite and proportional decrease in the rate of growth of the individuals.

8. X-radiation in *Simocephalus vetulus* of the intensities employed, produces also a prominent invagination over the upper portion of the brood pouch. This change is assumed to be the result of cellular distortion and changes in cell permeability.

9. On the basis of the foregoing assumption and from the observed decrease in the gaseous metabolism with the increase of intensity of X-radiation, it is concluded that X-rays produce injury of living cells and that the possible changes in cell permeability may control cellular metabolism.

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A CHANGE IN THE MORPHOLOGY OF A CLADOCERAN EFFECTED BY X-RAY TREATMENT[1]

(One plate)

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INTRODUCTION

IN A recent study, the rate of oxygen consumption of *Simocephalus vetulus* was determined after the organisms had been subjected to various doses of X-radiation (Obreshkove and King, 1932). Several hundred individuals were utilized in this study. The animals were subjected to X-radiation of known intensity immediately after their release from the brood chamber of the mother. The rate of oxygen consumption was measured 6 days later at a period corresponding to sexual maturity in the untreated individuals. It was shown that with an increase of the dose of X-radiation there is produced a corresponding decrease both in the rate of oxygen consumption and in the rate of growth of the individuals. In addition to the foregoing changes, X-irradiation of *S. vetulus* produces also prominent physical alterations in certain regions of the body. Without a single exception, X-rays of the type and the strength employed in this study produced a marked invagination over the brood pouch. This morphological change, once it appears, is retained in spite of the fact that with each shedding of the carapace there is produced a new and a larger covering enveloping the animal, which enables the rapidly growing animal to emerge from its previous crowded state. It is evident that this morphological change produced by X-radiation in *S. vetulus* must be the result of some physical, and perhaps some general, physiological alteration in the cells of the region involved. A brief account is given here of the effect of X-rays in producing a morphological change in *S. vetulus*. This work was done

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in the laboratories of the Department of Zoölogy and the Department of Radiology, Syracuse University. We wish to express our thanks to Dr. W. M. Smallwood, director of the Zoölogical Laboratories, for the many courtesies we received in the course of our research.

MATERIAL AND METHODS

The animals employed in this study represent the descendants of a single common mother, obtained from a pond in the vicinity of Poughkeepsie, New York. Since the animals were reared only parthenogenetically, they were all females, and the individuals of each new generation produced were genotypically identical.

The organisms were subjected to X-rays produced by a molybdenum target X-ray tube operated at 30 peak k.v. on a single-phase half-wave rectified-current power plant. The greatest portion of the energy under the foregoing conditions was emitted as relatively soft X-rays rich in the molybdenum Kα radiation with an average wave-length of approximately 0.712 Angstrom units (A). The tube was water cooled, and no filter was employed in this work. The animals were X-rayed in small groups. Usually ten individuals were placed in a depression slide containing a few drops of the culture media, and these were subjected to a beam of accurately measured intensity. The doses employed were 164, 251, 327, and 404 Roentgen units (r-units) per minute of irradiation and 1 hour exposure. The intensity was measured by a Victoreen Instrument Company ionization electrometer.

OBSERVATIONS

Figure 1 shows an untreated mature individual immediately after the release of its first brood of young. At this stage of the development, the animals are 6 days old and measure, on the average, 1.7 mm. in body length when reared at 25° C. and in a standard amount of the culture medium. The second clutch of young are seen deposited in the brood chamber. This chamber is relatively large; its dorsal surface and other tissues lying over it are seen to protrude prominently outward. This is in marked contrast, as we shall point out later, with the condition found in X-rayed individuals.

The skin of this species and of all Cladocera can be looked upon as

PLATE I

FIG. 1.—Untreated *Simocephalus vetulus*, 6 days old, immediately after the release of its first brood of young. The brood chamber is large, protrudes prominently outward, and contains a new clutch of eggs.

FIG. 2.—*Simocephalus vetulus*, 6 days old, X-irradiated immediately after its release from brood chamber with an intensity consisting of 327 *r*-units per minute and 1 hour exposure. The back of the animal is concave, and the cavity of the brood chamber is almost obliterated.

PLATE I

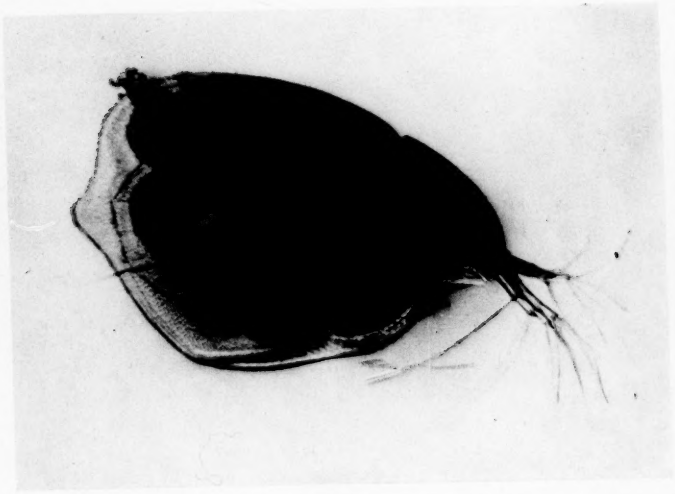

FIG. 1

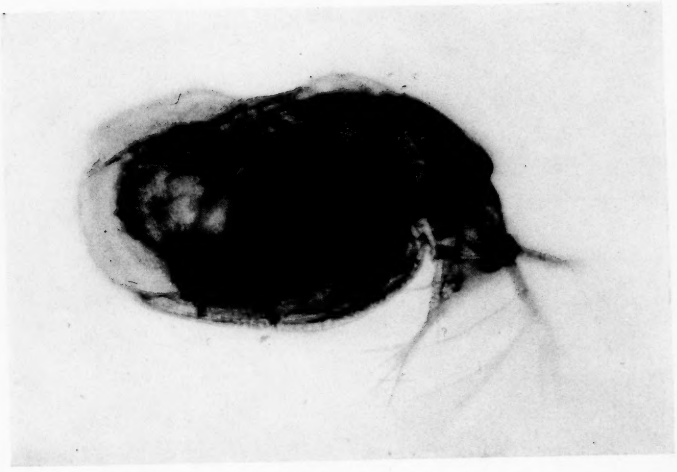

FIG. 2

consisting of two distinct layers. The tough shell, or the carapace, constitutes the outer of the two layers. Immediately underneath it, there is another more delicate and flexible layer. This layer contains formative cells which secrete the new carapace with each ecdysis. Between the outer and the inner layer of the skin, there circulates blood. It is held by some workers that this portion of the body has also a respiratory function. Each shedding of the carapace marks the beginning of a new instar in the development of the individual. Four such periodical sheddings are observed in the rapidly growing young before they reach sexual maturity. The inner upper surface of the brood chamber is lined with cells actively engaged in the secretion of a fluid. This fluid is poured into the chamber and serves as nourishment for the developing eggs and embryos.

Figure 2 shows an animal which had been subjected to 327 *r*-units of X-radiation per minute and 1 hour of exposure. The animal is 6 days old and represents a stage in the development which corresponds to sexual maturity in the untreated individuals. In this and all other animals subjected to X-radiation in this work, the radiant energy was applied immediately after the young were released from the brood chamber. At this stage the animals measure about 0.5 mm. in body length. The subsequent growth of the individuals after X-irradiation proceeds more slowly than in the untreated individuals.

An inspection of Figure 2 will reveal that X-radiation of the type and strength employed in this study produces other changes in *Simocephalus vetulus*. We observe that the dorsal portion of the brood chamber is prominently invaginated and that the cavity which in the untreated individuals is capable of accommodating a large number of eggs and developing embryos is now almost completely obliterated. There are no eggs in the brood pouch, and there is no evidence of developing germ cells in the ovary. These changes, particularly the presence of an invaginated brood chamber and a concave back, were found in every individual, without a single exception, when subjected to doses of X-irradiation varying in strength from 164 to 404 *r*-units per minute and 1 hour exposure. There appeared to be a relation between the degree of physical change produced and the strength of the X-rays applied.

The invagination over the brood chamber develops gradually. It

is not apparent in the young immediately after X-radiation, and it is not observed to exist on the following day. With the first shedding of the carapace, however, which occurs about 48 hours after the release of the young, there appeared a slight constriction over the dorsal portion of the brood chamber. With each subsequent ecdysis, the physical change produced becomes more and more prominent; and, once produced, it is retained till the death of the organism. This fact is of considerable interest, for, since the entire organism was subjected to X-rays, the brood pouch was still in contact with the organ systems for excretion, circulation of blood, and nutriment. No other morphological changes were observed in other portions of the body.

DISCUSSION

The morphological change produced in *Simocephalus vetulus*, represented by an invagination of the brood pouch and of the tissue lying over it, does not furnish us with sufficient data for an analysis of the dynamics of the processes involved. Since the entire organism was treated with X-rays and only a particular region of the entire body gave visible evidences of being affected, the observations recorded in this paper are of interest.

It is a well-known fact that different types of tissues possess different degrees of resistance to X-rays. We have observed in our work in connection with other problems that the developing eggs in the brood pouch of *S. vetulus* are more susceptible to X-rays than the germ cells in the ovary. On the other hand, a dose of X-radiation which proved lethal to the developing eggs in their early cleavage did not affect the embryos which were about to complete their differentiation. This apparent relation between the degree of sensitivity to X-rays and the cellular activity with regard to growth and development has been shown to hold true also in the case of the growing *Drosophila* eggs (Mavor, 1927). It is evident from the observations recorded in this paper that the definite relation which has been shown to exist between the activity and the resistance to X-rays may not apply only to cells which are undergoing growth and differentiation.

The upper portion of the brood pouch of *Simocephalus vetulus* and of all Cladocera is lined by very active glandular cells. These cells

secrete a fluid which is poured into the brood chamber and furnishes nourishment for the developing eggs and embryos (Birge, 1918). If our observations are correct, it appears that, next to the germ cells, these active secretory cells are more sensitive to X-rays than cells found elsewhere in the body, including the secretory cells of the inner layer of the skin. In *S. vetulus* each dose of X-radiation employed in the study produced an invagination of the upper portion of the brood pouch. The invagination of this portion of the body carried with it the tissues lying immediately over and closely adhering to it, producing in each individual subjected to X-rays a concave back. This physical change, once produced, is retained in the subsequent growth of the animal, although with each ecdysis a new and larger carapace was formed, enabling the animal to emerge from its previous crowded condition.

It is difficult at this time to arrive at definite conclusions in regard to the specific changes produced in the cells involved. The depression in the brood pouch and in the tissue lying over it cannot be looked upon as due to the absence of eggs in the chamber, for the change begins to appear long before eggs are deposited. It is possible, however, that the sterile condition of the ovary may have altered the physiology of the secretory cells at the upper portion of the brood pouch. And yet this condition has not been observed to exist in untreated individuals which fail to lay eggs for a long period of time or the ovaries of which were rendered sterile because of unfavorable laboratory conditions. It has been suggested that the physical changes described in the very active glandular cells of the brood pouch of *Simocephalus vetulus* are the result of changes in cell permeability due to X-irradiation. Osterhout (1912) holds that permeability is a relatively fixed property of the cell and that it changes as a result of injury. This contention appears very logical; and if correct, it still raises the question as to whether change in cell permeability is the primary cause for the disturbance and injury of X-irradiated cells. Nadson (1925) gives us a very fine picture of the progressive changes in yeast cells after exposure to radium. These changes include an increase in the number of fat droplets and an increase in the cytoplasmic granules, and in time the colloidal material of the cell becomes turbid. Undoubtedly, such events as these must

also serve as disturbing factors in altering and in injuring the normal properties of the cells. Packard (1931) holds that a change in permeability is a symptom of some physiological change in which the entire cell is involved.

SUMMARY

1. When *Simocephalus vetulus* young are subjected to X-rays of the type and strength employed in this study, there is produced in each individual a morphological change consisting of an invagination of the brood pouch.

2. This physical change develops gradually, and it becomes more and more prominent with each succeeding ecdysis.

3. It is suggested that the morphological change induced may be due to the changes of the permeability of the cells involved.

4. Since no other morphological changes were observed over other portions of the body, it is concluded that in this particular case the degree of resistance to X-rays is determined by the degree of cellular activity with regard to the secretory processes of the cells.

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SOME ASPECTS OF THE CAUSES OF NARCOSIS

(One figure)

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CHANGES PRODUCED BY NARCOTICS IN PROTOPLASM

THERE are several theories of narcosis, but none of them explains all peculiarities of this phenomenon. The only settled opinion concerning the causes of narcosis is the generally adopted opinion that these causes lie in some change inside the living content of the cells.

The best-known changes in protoplasm produced by narcotics were observed on plant cells. Narcotics bring about an interruption of protoplasmic movement in these cells and change the permeability and the viscosity of their protoplasm. Winterstein (1926) supposed that the interruption of protoplasmic movement in these cells produced by narcotics does not result from the anesthetic property of a narcotic but from its injurious effect. He pointed out that the concentration of narcotics necessary to produce this interruption is much greater than the concentration of the same narcotics in blood during anesthesia. It is well known, however, that even the cells of different nerve tissues of the same organism are differently sensitive to narcotics. It is therefore possible that plant cells require a greater concentration of narcotics to be transferred into the state of narcosis than nerve cells of animals. At the same time, a deep anesthesia in animals can lead to death, and it is unknown whether even a slight anesthesia is completely harmless.

It is well known that weak concentrations of narcotics decrease the permeability of protoplasm to salts and other substances insoluble in narcotics, while their greater concentration increases it. In one of the author's concurrently published papers (Lepeschkin, 1932) some experiments were described which showed that the decrease of

the permeability of protoplasm of *Elodea* leaves produced by ether and chloroform, is not accompanied by a cessation of protoplasmic movement; this movement is checked only by such concentrations of the narcotics which bring about an increase of the permeability or do not change it. Simultaneously with the changes of the permeability, changes of viscosity are observed (Heilbronn, 1914, 1922, Weber, 1922, 1925). A decreased viscosity corresponds to a decreased permeability, and vice versa. It is therefore not surprising that the speed of the protoplasmic movement increases according to the decreased viscosity at the concentration of narcotics which produce a decrease of permeability (Josing, 1901). On the other hand, it is comprehensible that an increase of the viscosity brings about a stoppage of the protoplasmic movement.

Winterstein (1926) explains this increase of viscosity by a suggestion that water is removed from protoplasm. But it is accompanied by an increase of the permeability of protoplasm to water-soluble substances; and this permeability depends, in the first place, upon the water content of protoplasm. The more water protoplasm contains, the more permeable it is for substances mentioned. Thus the only explanation of the increase of viscosity is the supposition that the volume of dispersed phases of protoplasm increases, due to a decrease of their dispersity or even to a slight coagulation in protoplasm. Narcotics can produce a coagulation in protoplasm because, owing to their low dielectric constant, they can produce it also in protein solutions (Lepeschkin, 1911; Freundlich and Rona, 1917; Loewe, 1913). The electric charge of colloid particles decreases if the dielectric constant of the surrounding medium is lowered. The coagulation produced by narcotics (for instance, by alcohol in protein solutions) is reversible at first but becomes irreversible afterward. This fact may explain the irreversible increase of viscosity in protoplasm at high concentrations of narcotics. The transformation of narcosis to death can be also explained. Every coagulation in protoplasm influences very unstable principal chemical compounds of protoplasm mechanically and causes them to decompose gradually (Lepeschkin, 1927*a*, *b*). If a sufficient amount of the principal compounds decomposes, no restoration is possible and the cells die. We may now indicate an answer to the question: "Which of all these

changes produced in protoplasm by narcotics correspond to the state of anesthesia of the animals which possess a well-developed nervous system?"

PROBABLE CAUSES OF ANESTHESIA

The above mentioned decrease of the permeability of protoplasm for salts, sugar, etc., produced by narcotics brings about an increase of the osmotic pressure in plant cells and therefore an increase of growth, which is experimentally proved by several authors (Winterstein, 1926). A simultaneous decrease of viscosity accelerates diffusion in protoplasm and therefore chemical reactions in it. Indeed, an accelerating effect of small concentration of narcotics on respiration of plant cells and other phenomena of metabolism is well established. An acceleration of protoplasm movement also assists the chemical reactions. To an increase of protoplasmic movements due to the decreased viscosity one should ascribe an accelerating effect of weak concentration of narcotics on phagocytosis, observed by Hamburger (1912). All these data support the interpretation that the decrease of permeability and viscosity and the acceleration of protoplasmic movement produced in plant cells by narcotics correspond to the stage of excitation of the narcosis of the animals possessing a well-developed nervous system.

On the other hand, an increase of permeability and viscosity, accompanied by the stoppage of protoplasm movement, decreases the osmotic pressure and growth of the cells and hinders chemical reaction in protoplasm. Moreover, a coagulation produced in the latter may make different substances dispersed in protoplasm and important for life inactive, especially such substances as enzymes. In this stage of the action of narcotics their concentration in protoplasm may increase so markedly that they displace substances important for respiration and adsorbed on the surface of dispersed phases of protoplasm, so that respiration stops completely (Warburg, 1921). Similar changes of the cell activity may also be expected to occur in the nervous system of animals. It is, therefore, more than probable that the increase of viscosity and permeability observed in plant cells under the influence of narcotics corresponds to anesthesia of higher animals. This implies that the causes of anesthesia are similar to the causes of these changes in protoplasm; in other words, an-

esthesia is brought about by a decrease of dispersity and coagulation of protoplasm colloids.

Bernard and Binz (cited according to Winterstein, 1926) were the first scientists who supposed that the state of anesthesia is brought about by a reversible coagulation in protoplasm. They observed a reversible turbidity in muscles and in the protoplasm of unicellular organisms which were brought into this state.

That an increase of dispersity and a coagulation of protoplasm colloids are the cause of anesthesia is also consistent with the facts that the effectiveness of a narcotic in producing anesthesia increases with a decreasing dielectric constant and with an increasing coagulating power (Winterstein, 1926), and that anesthesia is brought about not only by narcotics but also by other agents which produce a coagulation in protoplasm (mechanical influences, electric current, etc.) (Lepeschkin, 1924).

If our conception of the state of narcosis is correct, we may expect that:

1. The state of anesthesia would depend not merely upon the concentration of a narcotic in the surrounding medium but also upon the period of time during which this narcotic affects protoplasm of nerve cells, because every coagulation requires time.

2. The recovery from a deep narcosis would require much more time than that necessary for a removal of the narcotic from protoplasm. Indeed, a strong coagulation in protoplasm causes very unstable principal chemical compounds of protoplasm to decompose. Thus, a synthetic activity of cells is necessary in this case to restore the change produced in protoplasm.

3. Every kind of anesthesia, including even a slight one, would be injurious for organisms because even a slight coagulation influences the principal compounds of protoplasm mechanically so that they become more sensitive to the agents which produce their decomposition.

All these expectations proved to be indeed correct, as may be seen from the following experiments made on marine copepods.

EXPERIMENTS ON THE NARCOSIS OF COPEPODS

Marine copepods were used for these experiments in their natural grouping as they occur in plankton after the separation of immova-

ble animals and plants from it. Such plankton could be kept in constantly running sea water in good condition for some days. The outflow tube of the jar where the plankton was kept was provided with a small funnel covered by a plankton bolting cloth. The species of copepods used were defined by Professor Charles B. Wilson, and I express my hearty thanks to him for his aid. In most planktons used *Paracalanus parvus* was prevailing; while in some planktons the following species were found together: *Acartia clausi*, *A. tonsa*, *Oithona brevicornis*, *O. nana*, *Paracalanus parvus*, *Corycaeus rostratus*, *Oncaea venusta*, and *Euterpe acutifrons*. The experiments on copepods were made in the Scripps Institution of Oceanography in June and July, 1930; and I should like to express my thanks to the director of this Institution, Dr. T. W. Vaughan, for his kind support of my work.

Copepods are useful material for experiments on narcosis because of their small size and the great permeability of their surface tissues for narcotics. The constant swallowing of water by the animals also assists the penetration. The concentration of a narcotic in the surrounding sea water may be considered, therefore, as corresponding to the concentration of the same narcotic in the blood of Mammalia, in its action on the nervous system.

A suspension of copepods (*Paracalanus parvus* prevailing) in sea water was poured into a set of test tubes so that each tube contained about thirty animals in 8 cc. of sea water. Then different volumes of a saturated solution of ether, chloroform, or alcohol in sea water was added to each tube, and the time necessary to stop the movement of all animals in each tube was measured with a stop watch. The narcotics were obtained from Merck, carefully washed with water, dried and redistilled before experiments. The first action of the narcotics was excitatory; the movement of animals was accelerated for a short time; then it became slower and irregular, and finally stopped. This stage of the narcosis of copepods may be compared with the sleep stage of the narcosis of higher animals. During the first seconds of the sleep, the copepods reacted to mechanical excitation brought about by a shaking of the test tube or touching them with a glass hair, but afterward narcosis became complete and no stimulation produced any movement of the animals. In this stage of the narcosis, which may be called "anesthesia," the heart of *Paraca-*

lanus parvus stopped its beatings. But if the solution of narcotics was replaced by pure sea water, the heart began to beat first; then the animals executed some irregular movements, which became soon normal and normal life was recovered. The greater the concentration of narcotics, the quicker the anesthesia took place; and the more slowly the animals recovered after water had been substituted

TABLE I

DEPENDENCE OF THE TIME PERIOD IN WHICH ANESTHESIA AND RECOVERY TOOK PLACE UPON THE CONCENTRATION OF NARCOTICS

	Percentage of Concentration											
	Chloroform						Ether					
	0.2	0.07	0.04	0.018	0.009	0.005	3	2	0.88	0.42	0.2	0.08
Period of anesthesia in minutes..	0	0.01	0.04	0.25	55	∞	0	0.01	0.3	0.8	10	2,000
Period of recovery in minutes.....	∞	5	0.8	0.3	0.01	.....	∞	65	5	0.01	0	0

TABLE II

DEPENDENCE OF THE TIME PERIOD IN WHICH ANESTHESIA AND RECOVERY TOOK PLACE UPON THE CONCENTRATION OF ALCOHOL

	Percentage of Concentration					
	10	5.0	2.5	2	1	0.5
Period of anesthesia in minutes.....	0	0	0.01	25	120	∞
Period of recovery in minutes.....	∞	0.5	0.01	0	0	.....

for narcotic solutions. A further increase in the concentration of narcotics made recovery impossible and produced death. On the other hand, a certain low concentration made the movements of the animals temporarily slower but was not capable of producing anesthesia. This may be seen in Tables I and II. In these tables are given: the concentration of a narcotic in percentage (grams in 100 cc. of the solution), the period of time in minutes in which anesthesia took place, and the period of time in minutes in which the recovery of the animals took place after solutions of narcotics had been re-

placed by sea water in 10 minutes (chloroform), 20 minutes (ether), and 30 minutes (alcohol) after the addition of the narcotic. The symbol "∞" means that no anesthesia or recovery took place, while "0" means that it took place instantaneously.

These experiments show that the state of anesthesia depends not merely upon the concentration of a narcotic in the protoplasm of nervous cells but also upon the period of time during which the narcotic acts on the cells. Moreover, the recovery from the anesthesia cannot be merely ascribed to a removal of the narcotic by diffusion, because the size of animals is so small (smaller than 1 mm. long, and about 0.2 mm. thick) that one cannot think that the narcotics diffused out during 65 minutes. A synthetic activity of cells is evidently necessary to restore life after a deep narcosis. On the other hand, these experiments confirmed the opinion that a small increase of concentration of the narcotic transforms anesthesia to death. But even the concentrations of narcotics which produce a slight anesthesia lead to death if the anesthesia lasts for a sufficiently long time. Certainly, the period of time necessary to bring about death during anesthesia increases much more markedly with the decrease of the concentration of narcotics; 0.1 per cent solution of chloroform, for instance, kills the animals in 15 minutes, while 0.07 per cent solution of the same narcotic kills them in 2 hours. One may object, therefore, that in the case of comparatively small concentration of a narcotic, death results not from the action of anesthesia but from a cessation of some important physiological processes stopped by the anesthesia. It is easy to show, however, that even the slightest anesthesia which lasts for a very short time is harmful because it decreases the resistance of animals to poisons. On the other hand, a little smaller concentration which does not produce any anesthesia is harmless or even protective.

The period of time which was necessary to stop irreversibly the movement of copepods (*Paracalanus parvus*)—that is, to kill them—with a 0.0013 per cent solution of mercury bichloride, was defined first. This period was determined for five sets of tubes; each set contained five tubes, and each tube contained 8 cc. of sea water with about thirty animals. To the sea water of each tube was added 0.11 cc. of a 0.096 per cent solution of mercury bichloride. The average

period found for each of five sets of tubes was: 10.4, 11.0, 11.1, 10, and 10 minutes, or at an average 10.5 minutes. Then the necessary amount of a saturated solution of chloroform in sea water was added to new suspensions of animals so that the concentration of chloroform in the suspensions was 0.017 per cent, and anesthesia took place in 15 seconds. Immediately 0.11 cc. of the solution of mercury bichloride was added to the suspension in every tube, so that the concentration of mercury bichloride in the suspension was again 0.0013 per cent. After the animals had sunk to the bottom (the settlement was accelerated if necessary by a very slight centrifugalization which is completely harmless for the animals), the solutions in the tubes were replaced with sea water successively in 5, 6, 6.5, 7.5, and 8 minutes after the addition of mercury bichloride. The animals revived in the first, second, third, and fourth tubes, while they were dead in the fifth tube. Therefore, they were killed by mercury bichloride in 8 minutes. This experiment was repeated several times with the same result. If the concentration of chloroform in the suspension was 0.035 per cent (anesthesia took place in 5 minutes), 85 per cent of animals were killed by mercury bichloride in 6 minutes, and all animals were killed in 7 minutes. Thus it may be concluded that the resistance of the animals after they had been in the state of anesthesia for 7–8 minutes was noticeably decreased. If the concentration of chloroform was 0.008 per cent, no anesthesia took place in 5 minutes; and after the addition of mercury bichloride the animals were killed in 14 minutes. The results are given once more in Table III.

From these results it may be concluded that the state of anesthesia is harmful. We may suppose that the principal compounds of living matter are made more unstable by anesthesia, so that after a chemical change of their component parts produced by mercury bichloride they decompose easier than without preliminary action of narcotics. This decrease of stability can be ascribed to a mechanical influence of the coagulation brought about by chloroform. At all events, anesthesia is injurious for protoplasm and organisms, and it is gradually transformed to death. This conclusion is in accord with the fact that a narcotic is the more poisonous the more it is efficient in producing anesthesia (Winterstein, 1926).

We may now compare the dependence of the time period necessary to produce anesthesia upon the concentration of the narcotic with that of the time period necessary to produce a coagulation of proteins. The most suitable narcotic for the study of the coagulation

TABLE III

INFLUENCE OF CHLOROFORM ON THE RESISTANCE TO MERCURY BICHLORIDE

	PERCENTAGE OF CONCENTRATION OF CHLOROFORM			
	0	0.008	0.017	0.035
Anesthesia or not?	Not	Not	Yes	Yes
Period of time necessary to kill the animals with 0.0013 per cent of $HgCl_2$, in minutes	10.5	14	8	7

of proteins is alcohol, and the simplest protein to obtain is filtered white of eggs.

White of eggs, filtered through filter paper and completely clear, was poured into six test tubes, 8 cc. into each tube. Then 4 cc. of properly diluted ethyl alcohol was added to each tube under a constant shaking. The time period necessary to produce a turbidness in

TABLE IV

THE DEPENDENCE OF COAGULATION PERIOD UPON CONCENTRATION OF ALCOHOL

	PERCENTAGE OF CONCENTRATION OF ALCOHOL					
	22	21	20	19	18	16
Coagulation period in minutes	$\frac{1}{60}$	3.5	13	52	220	2,400

the white of eggs equal to the turbidness of a diluted solution of the same mixture of proteins in water, which had been heated to 100° C., is given in Table IV. Temperature, 25° C.

The similarity of the action of narcotics on copepods and that of alcohol on proteins may be seen especially distinctly in Figure 1, where the curves of this action are drawn. In the same figure the curve expressing the dependence of the survival time of copepods in solutions of mercury bichloride upon the concentration of the latter is given. This curve was recently obtained by the author (Lepesch-

kin, 1931). It is distinctly seen that this curve has a different character: it is smooth, while in the other curves the ascent is much steeper.

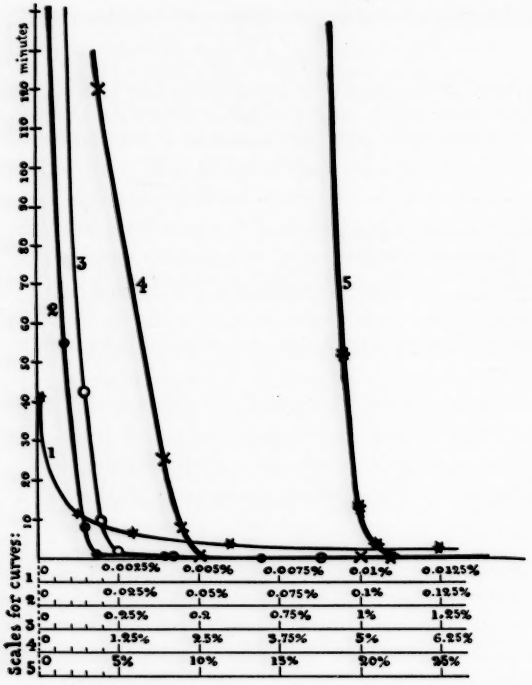

FIG. 1.—Curve 1 gives the dependence of the survival time of copepods on the concentration of mercury bichloride. Curves 2, 3, and 4 give the dependence of the time period necessary to produce anesthesia on the concentration of chloroform (2), ether (3), and alcohol (4). Curve 5 expresses the dependence of the coagulation time of white of eggs on the concentration of alcohol. The ordinates give periods of time in minutes and have the same scale for all curves. The abscissas give concentrations in percentage and have different scales for each curve.

This result confirms our supposition that anesthesia is brought about by a slight coagulation produced by narcotics in protoplasm. The action of poisons which do not produce any anesthesia is, however, different. Mercury bichloride, for instance, acts directly on the principal chemical compounds of protoplasm chemically; it reacts

with the protein part of these compounds and decomposes them. Certainly, the products of this decomposition may also coagulate, but only after the principal compounds have been markedly decomposed, so that no recovery is possible. The curve expressing the dependence of this action upon the concentration of mercury bichloride is a typical adsorption curve, because the molecules of proteins are thousands of times larger than those of mercury bichloride. The latter reacts, therefore, with protein molecules on their surface; they must be adsorbed before they can react.

SUMMARY

1. The state of anesthesia of higher animals corresponds to that stage of the action of narcotics on cells in which the permeability and viscosity of the latter increase and the protoplasmic movement stops. It is, therefore, very probable that a slight coagulation in the protoplasm of nerve cells produces anesthesia. This coagulation may remove substances important for life and thus stop physiological processes.

2. Every coagulation in protoplasm, and also the state of anesthesia, is harmful because they cause the principal chemical compounds of protoplasm to decompose. Marine copepods submitted to a slight anesthesia for a short time become less resistant to poisons (mercury bichloride), while chloroform concentrations which do not produce any anesthesia increase this resistance.

3. The curve expressing the dependence of the time period necessary to produce an anesthesia of copepods upon the concentration of the narcotics is similar to the curve of the dependence of the time period of protein coagulation upon the concentration of alcohol necessary to produce this coagulation. Both curves differ, however, from the curve expressing the dependence of survival time of copepods upon the concentration of mercury bichloride, in the suspension of these animals.

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